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<p>(21) International Application Number: <b>PCT/EP99/01678</b></p> <p>(22) International Filing Date: 12 March 1999 (12.03.99)</p> <p>(30) Priority Data:  98104479.5                      12 March 1998 (12.03.98)                      EP  99200439.0                      16 February 1999 (16.02.99)                      EP</p> <p>(71) Applicant (for AU BB CA CY GB GD GH IE IL KE LK LS MN MW NZ SD SG SZ TT UG only): <b>UNILEVER PLC (GB/GB); Unilever House, Blackfriars, London EC4P 4BQ (GB).</b></p> <p>(71) Applicant (for all designated States except AU BB CA CY GB GD GH IE IL KE LK LS MN MW NZ SD SG SZ TT UG): <b>UNILEVER N.V. [NL/NL]; Weena. 455, NL-3013 AL Rotterdam (NL).</b></p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): <b>CONVENTS, Daniel [BE/NL]; (NL) FRENKEN, Leon, Gerardus, Joseph [NL/NL]; Unilever Research Vlaardingen, Olivier van Noortlaan 120, NL-3133 AT Vlaardingen (NL). HARMSEN, Michael, Marie [NL/NL]; ID-DLO, Edelhertweg 15, NL-8219 PH Lelystad (NL). VAN DER LINDEN,</b></p>		<p>Richard, Henricus, Jacobus [NL/NL]; Universiteit Utrecht, Heidelberglaan 8, NL-3584 CS Utrecht (NL). VERRIPS, Theodorus, Cornelis [NL/NL]; Unilever Research Vlaardingen, Olivier vanNoortlaan 120, NL-3133 AT Vlaardingen (NL).</p> <p>(74) Agent: <b>JOPPE, Hermina, L., P.; Unilever NV, Patent Dept., P.O. Box 137, NL-3130 AC Vlaardingen (NL).</b></p> <p>(81) Designated States: <b>AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</b></p> <p><b>Published</b>  <i>With international search report.  Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: <b>PRODUCTS COMPRISING INACTIVATED YEASTS OR MOULDS AND ACTIVE VHH-TYPE ANTIBODIES</b></p> <p>(57) Abstract</p> <p>The use of VHHs<sup>6</sup> in the preparation of products to provide stability of antibody specificity under destabilising conditions whereby normally lower eukaryotes or traditional antibodies are killed or inactivated.</p>		

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## PRODUCTS COMPRISING INACTIVATED YEASTS OR MOULDS AND ACTIVE VHH-TYPE ANTIBODIES

### Field of the Invention

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The present invention is in the field of applied biotechnology and relates in particular to an economic way of introducing antibodies into a variety of products, especially consumer product such as food products and  
10 personal care products and animal feed.

### Background of the Invention

To fulfil many unmet demands of the society, there  
15 is a clear need to provide new products from which consumers may benefit, in particular in the field of food products including animal feed and personal care products such as laundry detergents, deodorants etc. One of the problems with the presently used consumer products is that  
20 if they contain functional compounds, these compounds are often not very specific.

For instance, for killing microorganisms in food products the functional compound may be a salt or an acid.  
25 If the concentration of either of these compounds is sufficiently high, they are able to kill microorganisms. However, often the level of salt renders the taste of the product less attractive to the consumer. Moreover, high levels of salt are not recommended by health authorities.  
30 The same holds for products that contain considerable amounts of acids or preservatives.

Non-food consumer products such as personal care products e.g. laundry detergent products may contain compounds that are able to bleach stains, but as they do not specifically recognise stains, considerable amounts of these bleach generating compounds (e.g. percarbonate/TAED) are necessary.

Another example is that some hair care products contain compounds that kill the microorganisms involved in dandruff. Also these compounds are not specific and in addition to the causative microorganisms of dandruff they also kill other microorganisms on the skin which are beneficial to the consumer.

Still another example of an unmet demand of the society is the overproduction of manure due to cattle farming. Part of this problem is that the conversion of animal feed into nutrients is not optimal due to the presence of negative compounds like phytic acid.

The above given examples of unmet consumer needs and society needs can be extended, but it will already be clear that consumer products and animal feed with compounds which specifically meet the demand of the consumer or society will be of great importance.

The main characteristic of processes in living cells or living species is that they are often highly specific. Enzymes only recognise specific substrates and the specificity of antibodies is unsurpassed. Therefore in recent years the industries involved in the manufacturing of (chemical and biotechnological) consumer products or

animal feed are increasingly applying biological molecules in order to make their products more specific.

A successful example of this development is the  
5 introduction of proteases, lipases, cellulases and amylases  
in laundry products. These enzymes have a certain  
specificity and because the production of these enzymes has  
been improved considerably by recombinant DNA techniques,  
these microbial enzymes can be produced by microorganisms  
10 at costs affordable for consumer products. The introduction  
of enzymes in detergents has indeed met demands of our  
society. Over the last decades the energy usage for  
cleaning of laundry has been reduced with about 50% and the  
amount of chemicals with about 40%.

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In the area of animal feed enzymes can play an  
important role to reduce the environmental pollution, as  
has been nicely demonstrated by the application of the  
enzyme phytase in animal feed. Again this development was  
20 possible due to the enormous improvement of the production  
of phytase by rDNA techniques.

However, often a higher specificity than just  
recognising a protein or fat is highly desirable to fulfil  
25 the unmet demands and therefore the consumer goods and  
animal feed industry is looking for ways to get  
specificities in the same order as antibodies have.

In nature microorganisms do not produce antibodies.  
30 Although it is well known that by using rDNA technology  
microorganisms can produce almost any protein, irrespective  
of its origin, the yield of homologous proteins is much

higher than for heterologous proteins. Moreover, expensive purification processes are needed to recover the heterologous protein from its producing cell, since it is not allowed to bring living rDNA organisms into the environment. These two factors, the low production yield and the fact that rDNA organisms have to be separated from the products that they produce have slowed down the introduction of heterologous proteins in consumer products other than in laundry products and some food products. In animal feed only the addition of phytase is a real success up to now.

The present invention relates in particular to introducing certain categories of antibodies into a variety of products, especially food products, personal care products and animal feed.

Hamers-Casterman et al., *Nature* (1993) **363**:446-448 disclose isolated immunoglobulins from the serum of *Camelidae* comprising two heavy polypeptide chains sufficient for the formation of a complete antigen binding site, which immunoglobulins further being devoid of light polypeptide chains.

WO 94/25591 discloses the production of antibodies or functionalised fragments thereof derived from heavy chain immunoglobulins of *Camelidae*, using transformed lower eukaryote host organisms.

Spinelli et al., *Nature structural biology* (1996) **3**:752-757 disclose the crystal structure of a llama heavy chain variable domain.

In many processes involving recombinant DNA techniques and culturing or fermenting transformed microorganisms, it is necessary at some stage, for example at the conclusion of the fermentation, to kill the active cells in order to prevent any viable recombinant organisms from being released into the environment.

A conventional way of killing cells is using heat. U.S. Patent 4,601,986 is an example of the use of heat to kill the cells and stop the growth of microorganism cultures. Other conventional ways of killing cells are by lysing the cells, for example by applying high osmotic pressures or by adding enzymes which break down the cell walls or membranes. These techniques are exemplified in U.S. Patents 4,299,858, 3,816,260, 3,890,198, and 3,917,510.

In many systems host microorganisms, for example lower eukaryote cells, are difficult to kill. Conventional methods, such as heat, are too severe and may destroy or alter the desired product before the cells are killed. This applies also for the immobilized systems of binding proteins on lower eukaryotes disclosed in WO 94/18330, when the binding protein is an antibody or a functional fragment thereof.

It is often desirable to manufacture, store or use products under conditions which would normally be detrimental to the stability of biological systems such as microorganisms or antibodies. For example products may desirably be heated, e.g. pasteurised during manufacture

or use (e.g. high temperature washing) or its composition and/or pH may be such that the biological systems do not survive storage over pro-longed periods.

5           Similarly, there is still a need for stable functional systems possibly involving microorganisms, where the microorganisms are inactivated or killed and/or their growth is suppressed while the functional activity of the system is substantially maintained.

10           In this light there is a need to use components in products, especially consumer products, whereby on the one hand said components have a acceptable or even good specificity and on the other hand are stable under  
15 conditions of manufacture and/or storage and/or use.

#### Brief description of Terms

          "As, used herein, the term "antibody" refers to an  
20 immunoglobulin which may be derived from natural sources or may be synthetically produced, in whole or as antibody fragment.

          An "antibody fragment" is a portion of a whole  
25 antibody which retains the ability to exhibit antigen binding activity. Functionalized antibody fragments are also embraced in this term.

          The term "functionalized antibody fragment" is used  
30 for indicating an antibody or fragment thereof to which one or more functional groups, including enzymes and other binding polypeptides, are attached resulting in fusion



products of such antibody fragment with another biofunctional molecule.

The term "traditional antibody" is used for an antibody which normally consists of two heavy and two light chains or fragments thereof.

The term "VHH" refers to the single heavy chain variable domain antibodies of the type that can be found in Camelid mammals which are naturally devoid of light chains; synthetic and naive VHH can be construed accordingly.

#### Summary of the Invention

15 In one aspect the present invention relates to a method of preparing, storing or using a product comprising active antibodies, wherein said method involves the application of conditions whereby normally lower eukaryotes or traditional antibodies are killed or inactivated and  
20 whereby the active antibodies are VHHs.

In another aspect the invention relates to the use of VHHs in the preparation of products to provide stability of antibody specificity under conditions whereby normally  
25 lower eukaryotes or traditional antibodies are killed or inactivated.

These and other aspects of the invention will be described in further detail in the description which  
30 follows.

### Brief Description of the Figures

Figure 1 shows a typical dilution curve for the antibodies used, from which the sub-saturation point was 5 determined.

Figure 2 shows the antigen binding after temperature treatment of hCG specific mouse MAbs (A) and llama V<sub>HHS</sub> (B) or RR-6 specific mouse MAbs (C) and llama V<sub>HHS</sub> (D). Antibodies were incubated for 2 hrs at different temperatures, cooled down to room temperature and subsequently ELISA was performed.

Figure 3 shows the antigen binding in presence of 15 ammonium thiocyanate at sub saturation point of hCG specific mouse MAbs (A) and llama V<sub>HHS</sub> (B) or RR-6 specific mouse MAbs (C) and llama V<sub>HHS</sub> (D).

Figure 4 represents a restriction map of plasmid pUR 4482

Figure 5 represents a restriction map of plasmid pUR 4588.

Figure 6 represents a restriction map of plasmid pUR 4676

Figure 7 represents a restriction map of plasmid pUR 4659

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Figure 8 shows the influence of different levels of OMO (without protease) on the stability of anti-RR120 V<sub>HHS</sub>

in 25 mM NaCO<sub>3</sub> and pH 10.3, whereby the amount of binding in 25 mM NaCO<sub>3</sub> at pH 10.3 is set at 100% immunoreactivity.

Figure 9 shows the influence of different levels of 5 LAS on the stability of anti-RR120 VHs in 25 mM NaCO<sub>3</sub> and pH 10.3, whereby the amount of binding in 25 mM NaCO<sub>3</sub> at pH 10.3 is set at 100% immunoreactivity.

Figure 10 shows the amino acid sequence of anti-10 RR120 VHs A38 (SEQ ID:NO 23) and A44 (SEQ ID:NO 24) (obtained by screening in PBS), A306 (SEQ ID:NO 25), A307 (SEQ ID:NO 26) and A308 (SEQ ID:NO 27) (obtained by screening in LAS) and A309 (obtained by screening in Dutch Omo without protease; SEQ ID:NO 28).

15

#### Detailed Description of the Invention

Traditional antibodies, consisting of two heavy and 20 two light chains are rather stable when they circulate in the blood and lymph systems to protect the host against invaders. This stability is caused by several S-S bridges between the heavy chains and the strong interaction between the heavy and the light chain. However, production of 25 complete antibodies in microorganisms is very difficult and economically not yet feasible for introduction into consumer products or animal feed.

Recently, it has been shown that antibody fragments 30 of traditional antibodies can be produced by certain microorganisms, but in general the yield of these fragments is rather low. The stability of fragments of traditional

antibodies is rather low and the physics of protein stability is still not sufficiently developed to predict protein stability from its amino acid sequence.

5           The present invention is based on the surprising finding, after extensive research and experimentation, that on average VHHs are more stable against destabilising physical and/or chemical conditions such as one or more of:  
high temperature, ultra high pressure, pulse electric  
10 field, radiation, presence of anti-microbial, presence of (mixed) surfactants, presence of ethanol, pH altering materials and presence of chaotropic agents as compared to eukaryotic cells or as compared to traditional antibodies. It is even more surprising that certain variable domains of  
15 Camelidae antibodies from llamas appear to be very stable under pasteurisation conditions.

Therefore in a first aspect the inventions relates to a method of preparing (including the identification of  
20 antibodies), storing or using a product comprising active antibodies, wherein said method involves the application of destabilising conditions whereby normally lower eukaryotes or traditional antibodies are killed or inactivated and wherein the active antibodies are VHHs.

25

In another aspect the invention relates to the use of VHHs in the preparation of products to provide stability of antibody specificity under destabilising conditions whereby normally lower eukaryotes or traditional antibodies  
30 are killed or inactivated.

The invention is further based on the finding that physical techniques or chemical treatment, or a combination of physical techniques and chemical treatment or a combination of special types of mild techniques which are destabilising conditions in that they are able to kill or inactivate lower eukaryotes e.g. *Saccharomyces Cerevisiae*. Typically the lower eukaryotes would be reduced by a factor of at least  $10^6$ , or which are able to kill or inactivate traditional antibodies, surprisingly can be used whereby the VHHs retain their immunoreactivity to a large extent, typically >60%, and preferably from about 70% to 100% or even 90% to 100%.

Preferably the destabilising conditions are such that normally lower eukaryote cells are inactivated with a factor of at least  $10^6$ . Typically the conditions are chosen such that under these conditions lower eukaryotes would be reduced by a factor of at least  $10^6$ , because this killing efficiency is often acceptable for the health authorities as killing efficiency for pathogenic bacteria in food products. A preferred reduction is with a factor of  $10^7$  to  $10^9$ . Particularly the conditions are chosen such that the activity of *Saccharomyces cerevisiae* would be reduced by a factor of at least  $10^6$ .

25

In a preferred embodiment the destabilising conditions are such that normally the immunoreactivity of traditional antibodies is reduced to less than 70%, more preferred less than 10%, e.g. less than 1% or even less than 0.01%. Typical examples of traditional antibodies are mouse monoclonal antibodies. Especially preferably the destabilising conditions are such that the immunoreactivity

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of mouse monoclonal antibodies against hCG as described in Gani M, Coley J and Porter P Hybridoma 6: (6) 637-643, Dec 1987 (hereafter referred to as HCGMABS) is reduced to less than 70%, more preferred less than 10%, e.g. less than 1% or even less than 0.01%.

The VHHs for use in accordance to the invention are stable under the conditions whereby the lower eukaryotic cells or traditional antibodies are normally killed or substantially inactivated. In particular they preferably recognise their binding target e.g. harmful microorganisms or toxins with a binding constant of  $>10^4$ , preferably  $>10^6$  or  $>10^7$ , most preferably  $>10^8$  and/or retain their immunoreactivity for at least 70%, more preferred from 80-100%, most preferred from 90 to 100% after application of the destabilising conditions for eukaryotic cells and/or traditional antibodies.

The destabilising conditions preferably comprise physical treatments and/or chemical conditions which are applied during manufacture, storage or use of the product.

Suitable examples of physical treatment as destabilising conditions are selected from one or more of:

(a) a high temperature treatment, for example at more than 70 °C, for example 80°, 90° or 100 °C, especially 70-100 °C. The duration of the heat treatment is not very critical but will normally be between 30 seconds and 2 hours, e.g. 1-60 minutes. Preferably the duration is such that on the one hand destabilising conditions are present for lower eukaryotes or traditional antibodies, but on the other hand the VHHs are not substantially deactivated.

Typical examples of such conditions are for example pasteurisation of the product during manufacture or use of the product under high temperature conditions e.g. high temperature (e.g.  $>70^{\circ}\text{C}$  washing).

5 (b) ultra high pressure treatment, for example at 250 Mpa or more, say 250-500 Mpa. Again the duration of the high pressure treatment is not very critical, but will normally be between 1 second and 2 hours, e.g. 1-60 minutes.

10 (c) irradiation treatment, for example at least 10 kGray gamma radiation, for example 10-50 kGray gamma radiation.

(d) pulse electric field treatment. Suitable application conditions of pulse electric field are well-  
15 known in the art.

Optionally physical treatment destabilising conditions can be used in combination with one or more chemical destabilising conditions. If this is done,  
20 generally it is possible to use relatively mild physical conditions in combination with relatively mild chemical conditions. In particular it was found that combinations of mild physical treatments and antimicrobial compounds, such as acids or antimicrobial peptides were even more effective  
25 to kill the lower eukaryotes, while maintaining the functionality of the VHH. Using these combinations, killing effects of  $>10^7$  could be achieved, while the functionality of the antibody remained substantially unaffected.

30 In another embodiment of the invention the destabilising conditions involve chemical treatment e.g. selected from one or more of:

(a) the presence of an effective amount of antimicrobial agent, for example sorbic acid, benzoic acid, nisin, MB21, or another bacteriocin. Typically the effective amount of antimicrobial agents are chosen such  
5 that they are capable to contribute to the destabilising conditions. The exact effective level is usually dependent on the type of agent and the whether or not other destabilising treatments are applied but may normally be from 1ppm to 5 wt%, e.g. 0.01 to 2 wt%.

10 (b) the presence of an effective amount of a cell wall degrading enzyme, for example  $\beta$ -(1,3)-glucanase,  $\beta$ -(1,6)-glucanase, chitinase and/or a redox enzyme. Typically the effective amount of such enzymes are chosen such that they are capable to contribute to the destabilising conditions.  
15 The exact effective level is usually dependant on the type of enzyme and whether or not other destabilising treatments are applied but may normally be from 1 ppm to 1 wt%, e.g. 0.0001 to 0.1 wt%.

(c) the presence of solvent materials e.g. alcohol at  
20 for example at a level of at least 10 wt%. These high levels of alcohol are often destabilising especially for lower eukaryotes such as yeasts. Typical destabilising levels for solvent materials may vary in a wide range dependant on the type of solvent and the other  
25 destabilising conditions which are applied. Normally levels of 3- 96 wt% would be used, e.g. 10 to 35 wt% of the product. A convenient solvent material is ethanol.

(d) the presence of surfactant materials, especially mixtures of two or more surfactants e.g. selected from the  
30 group of anionic, nonionic or zwitterionic detergency materials for example at a level of at least 0.2 g/l. Typical examples of suitable surfactant materials are well-



known in the art e.g. in the formulation of household detergent or personal washing systems such as shampoo. The level of the surfactant materials may vary e.g. between 5 and 50 wt% of the product, e.g. 10-30 wt% or much lower levels such as 0.01 to 20 g/l in the washing solution or the screening medium. Advantageously, mixtures of anionic and nonionic surfactants are used in a weight ratio of from 10:1 to 1:10.

(e) the presence of chaotropic materials e.g. ammonium thiocyanate, ureum and/or ATC elution;

(f) the presence of materials e.g. acidifying or buffering materials which render the pH less than 5 or more than 9, examples of these are  $\text{NaCO}_3$ , acetic acid, citric acid etc.

15

As described above the chemical treatment may advantageously be used in combination with one or more physical treatments.

20 In accordance to an aspect of the invention the destabilising conditions are applied during one or more of preparation, storage or use of the product. For the purpose of this invention the term "preparation of the product" refers to all steps carried out in the preparation of the  
25 product in the presence of VHH such as on the one hand "selection" of the VHH, and on the other hand actual "manufacture" of the product e.g. mixing of ingredients with VHHs, pasteurization, drying, packaging etc.

30 Surprisingly it has been found that VHHs are often more stable than traditional antibodies. One of the problems in the manufacture of products especially the

stage of identification of suitable antibodies for use in products e.g. food, personal care or animal feed products is that the selection is usually done in a model system.

The good stability of VHHs now renders it possible to carry  
5 out the identification of the effective VHHs under destabilisation conditions. For example the VHHs can be screened in a medium comparable to the product in which the VHH is to be used. For example antibodies for use in personal care products such cleaning formulations e.g.  
10 laundry detergents or shampoos can be screened in the product. This is an easy and effective way to identify VHHs which on the one hand have adequate binding affinity towards the target molecule, but which are on the other hand stable under product conditions.

15

Although applicants have found that VHHs are on average more stable than traditional antibodies or lower eukaryotes, it may still be desirable to apply a selection  
step to VHHs whereby the VHHs which are most stable under  
20 the destabilising conditions are selected. Such a selection step may be carried out by any suitable method, however as described above a preferred method involves the screening of VHHs under product conditions e.g. in the presence of a system which is equal or similar to the product in which  
25 the VHH is to be used. Therefore in a preferred embodiment of the invention both the selection of suitable VHHs and at least one further step selected from manufacture, use or storage takes place under destabilising conditions.

30 Therefore the invention also relates to a method to prepare a product comprising VHHs, comprising the steps of:

selection of suitable VHHs by screening under conditions which are equal or similar to the conditions of the product; and incorporating the selected VHHs in the product. Preferably such product is manufactured, used or  
5 stored under destabilising conditions for lower eukaryotes or traditional antibodies as described above. The invention also encompasses a product obtainable by such a method.

In another embodiment of the invention, the  
10 manufacture of the product involves the combination of ingredients and application of destabilising conditions. For example it is often desirable in the production of a food, personal care or animal feed product to heat the product, e.g. to ensure pasteurisation to ensure  
15 keepability. If traditional antibodies are present during such a heating step, they are generally inactivated and hence they can only be added after the pasteurization step, which is clearly less desired. Surprisingly it has been found that VHHs are often more stable against the use of  
20 destabilising conditions applied in the manufacturing process of products.

In another embodiment of the invention the storage of the product involves the application of destabilising  
25 conditions. For example the chemical composition of the product may contain ingredients which would normally destabilise traditional antibodies and/or eukaryotes. Surprisingly it has been found that VHHs have an enhanced stability wrt said destabilising conditions which renders  
30 it possible to store the product for pro-longed periods e.g 1 week to 1 year without an unacceptable reduction of immunoreactivity.

In another embodiment the use of the product involves the application of destabilising conditions. For example the use of a food product may include the heating thereof to temperatures which would normally destabilise lower eukaryotes and/or traditional antibodies. Alternatively personal care products e.g. laundry detergents may be used under high temperature conditions. Surprisingly it has been found that VHHs can be used and retain their stability for the antibody activity when destabilising conditions are applied during use.

Preferred products in which VHHs can be applied include, for example, consumer products. A preferred group of products are food products such as ice-cream, oil based products such as margarines, oils, mayonnaise, dressings, soups, sauces, soft drinks, for example tea based drinks, meals etc. Another preferred group of products are personal care product such as cleaning products like soap bars, washing liquids, gels, laundry detergents e.g. in powder, paste, tablet or liquid form, and deodorants, creams, lotions, shampoo etc. A third group of products are animal feed products in dry or wet form.

Typical amounts of VHHs in the product are from 0.001 $\mu$ M to 1 M, under the destabilising conditions e.g. in the preparation, storage or use the amounts can typically be from 0.1 to 100  $\mu$ M, e.g. 2 to 20  $\mu$ M.

The invention will be illustrated by means of the following examples which also show the use of Mouse monoclonal antibodies.

Mouse monoclonal antibodies (MAbs) or fragments thereof have many potential applications in addition to for example cancer therapy and diagnostic kits. However, for 5 most applications large quantities are needed whereas costs have to be reduced. For MAbs or fragments thereof these conditions often cannot be met. The *Camelidae* heavy-chain antibodies offer a solution to this problem. Probably because of their simple one chain structure and their 10 solubility (Spinelli et al., 1996), VHHs, especially the variable part of llama heavy chain can be secreted relatively pure and in high amounts by the yeast *S. cerevisiae*. For this reason VHHs, especially llama VHHs are suitable for large scale applications.

15

As will be shown in the experimental part, VHHs, especially llama VHHs are far more heat stable compared to mouse MAbs. Most VHHa were still able to bind after two hours at temperatures as high as 90°C. The thermal 20 denaturation at lower temperatures of mouse MAbs can be explained because of heavy and light chain separation. After cooling down, these fragments will not associate, or only randomly, resulting substantially in non-functional antibodies.

25

In general, VHHs and mouse MAbs are comparable in antigen binding in the presence of ATC. HCG specific antibodies could bind at higher ATC concentrations than RR-6 specific antibodies. This is probably due to the nature 30 of the antigen and or antibody-antigen interaction.

Regarding the specificity, VHHs have about the same potential as mouse MAbs in recognition of antigen. Within the small subset of anti-hCG, llama V<sub>HHS</sub>, some were found specific to separate alpha-subunit, beta-subunit and intact hCG. The anti-RR6 llama V<sub>HHS</sub> did not crossreact with other azodyes, resembling RR-6 in structure. The small subset tested seems to be very specific to RR-6.

Most of the VHHs have apparent affinities ( $K_D$ ) range of  $10^{-7}$ - $10^{-9}$  M, which is of high affinity. Some mouse MAbs used were found to have higher affinity ( $10^9$ - $10^{11}$  M<sup>-1</sup>) which can be contributed to avidity because mouse MAbs are bivalent.

In summary, when comparing the biochemical properties of VHHs, especially llama VHHs and mouse MAbs for stability, specificity and affinity, it appears that VHHs have physical chemical properties which make them excellent candidates for use both in existing and novel applications. These applications are in a variety of fields and which are immediately evident to the man skilled in the art. As most food products have to be pasteurized, the addition of VHHs create really new options. For example, VHHs that bind and neutralize redox enzymes thereby preventing colour changes of food products, or VHHs that recognize proteins or polysaccharide of food products thereby providing or improving the structure to the product, or VHHs that bind off flavours (scavenging) or bind flavours (controlled release).

The invention is further illustrated by the following experimental work which however is not intended to limit the invention in any respect.

**Example I: Materials and methods**

To investigate if V<sub>HH</sub>s such as llama antibodies can be used in a range of applications, a number of physical  
5 chemical and functional characteristics was investigated, thereby concentrating on three characteristics: stability, specificity and affinity. Llama V<sub>HH</sub>s and "classical" bivalent mouse monoclonal IgG antibodies ("mouse MAbs"), specific for either the human pregnancy hormone or the  
10 azodye Reactive Red-6 ("RR-6"), were compared with respect to heat stability and antigen binding in chaotropic environment (ammonium thiocyanate, ATC elution). Furthermore, llama V<sub>HH</sub>s were tested for their antigen specificity and affinity. Both llama and mouse antibodies  
15 consist of two subsets: one specific for the protein antigen human Chorionic Gonadotropin (hCG) and one specific for the hapten azodye RR-6.

The results show that llama especially regarding  
20 functional binding at high temperature (about 90°C) llama V<sub>HH</sub>s are extraordinarily stable, compared to mouse MAbs.

**Example I.1 Purification of llama V<sub>HH</sub>s and mouse MAbs**

25 Llama V<sub>HH</sub>s HI-6, H-13, H-14, HI-15, HI-113 (anti-hCG) and R2, R4, R7, R8, R9, R10 (anti RR-6), described in EMBL data library accession numbers: AJ236095 (HI-6), AJ236096 (H-13), AJ236094 (H-14), AJ236097 (HI-15), AJ236098 (HI-113), AJ236100 (R2), AJ236102 (R4), AJ236105  
30 (R7), AJ236106 (R8), AJ236107 (R9), and AJ236108 (R10), were secreted by *S. cerevisiae* as a fusion protein comprising a V<sub>HH</sub> with a C-terminal myc-tag and a (His)<sub>6</sub>-tag.



For stability studies, yeast culture supernatants containing llama  $V_{HH}$ s were ultrafiltrated using membranes with cut off limits of 50kD and 5kD, respectively, (Filtron, USA) and dialysed against phosphate buffered 5 saline (PBS, 145 mM NaCl, 7.5 mM  $Na_2HPO_4$ , 2.5 mM  $NaH_2PO_4$ , 0.01% sodium azide).

The final  $V_{HH}$  preparation had a purity between 80-90% as judged by Coomassie Brilliant Blue (R-250, Sigma, 10 Zwijndrecht, the Netherlands) staining of sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE).

Mouse MAbs were obtained from hybridoma culture supernatant and purified using protein A and subsequent 15 dialysis against PBS. The hCG specific mouse MAbs used was mAB 3299 (anti-Thyroid Stimulating Hormone). The RR-6 specific mouse MAbs used was URL 08-04.

#### **Example I.2 Sub-saturation point**

20

Using ELISA, dilution curves were made for all antibodies. From these dilution curves subsaturation point was determined (Fig. 1). The sub-saturation point was defined as the highest concentration on the linear part of 25 the curve. Using this sub-saturation point any effect on antigen binding can be observed as a decrease in signal.

**Example I.3 Temperature**

Llama  $V_{HH}$ s and mouse MAbs were incubated at various temperatures (4°C, 60°C, 70°C, 80°C and 90°C) for two hours. Subsequently, antibodies were put 30 minutes at 25°C and stored at 4°C. Samples were diluted to sub saturation point and ELISA was performed (Fig. 2). It can be concluded that the  $V_{HH}$  fragments are more stable than the MAbs.

10

**Example I.4 Binding in the presence of Ammonium Thiocyanate**

Double ATC (0-8M) concentrations were mixed 1:1 with double sub saturation concentrations of antibodies and were incubated for 10 min at room temperature. Standard ELISA was performed using antibody-ATC mixtures. See Figure 3.

From Figure 3B and 3D it can be concluded that the llama  $V_{HH}$  fragments HI-15 and R7 are able to bind at higher concentrations ammonium thiocyanate than any of the RR-6 or hCG specific mouse MAbs tested. The binding of HI-15 was reduced only 20% in 4M ammonium thiocyanate, whereas none of the other antibodies tested were able to bind at this concentration. Llama  $V_{HH}$  fragment R7 was completely inhibited in RR-6 binding at 4M ammonium thiocyanate, whereas the best anti RR-6 Mabs (URL 08-01 and URL 08-13) were already completely inhibited at 2M ammonium thiocyanate (Fig. 3C).

30

Based on these findings applicants are of the opinion that it is well within the ability of the skilled

person to find VHHs which are stable under conditions which are normally destabilising for traditional antibodies such as Mouse Mabs.

### 5 Example I.5 Specificity of llama V<sub>HHS</sub>.

ELISA was performed on separate alpha-, beta-subunit, or native hCG. The results are shown in Table 1.

10 Table 1

#### Specificity llama V<sub>HHS</sub> anti-hCG

Llama V <sub>HH</sub>	Alpha	Beta	Intact
HI-6	-	+	+
H-13	+	-	+
H-14	+	-	+
HI-15	+	-	+
HI-113	-	-	+

15

Within this small subset, llama V<sub>HHS</sub> were found specific to separate alpha, beta, or native hCG. This indicates that llama V<sub>HHS</sub> have the same potential as mouse MAb in recognition of antigen.

**Example I.6 Affinity**

Apparent affinities of a selection of llama  $V_{HH}$ s were determined by kinetic measurements using the IAsys Biosensor (Affinity Sensors, Cambridge, UK). The results are given in Table 2 below.

Table 2: Affinity values llama  $V_{HH}$ s and mouse MAbs

10

Llama $V_{HH}$	Kd (nM)	Mouse Mab	Kd (nM)
H-14	300-400	URL 08-04	1
R2	22	Fab URL 08-04	8.4
R7	45	Mab 3299	3
R8	20		
R9	83		
R10	58		

**Example II.1 - Induction of humoral immune responses in llama**

A male llama was immunized with K88ac fimbriae, also known as F4 fimbriae, which were purified from *E. coli* strain 1087 according to Van Zijderveld et al. (1990). Immunizations were performed both subcutaneously and intramuscularly using 1 ml 50 mg/l K88ac per immunization site. The first two immunizations were performed with a three week interval and using a water in oil emulsion (4:5 (v/v) antigen in water:specol) as described by Bokhout et al. (1981 and 1986). The third and fourth immunization were done without adjuvant, five and nine weeks after the first immunization. The immune response was followed by antigen specific ELISA's.

Polystyrene microtiter ELISA plates (Greiner HC plates) were activated overnight at 4°C with 100 µl/well of 5 mg/l K88ac antigen in 0.05 M sodium carbonate buffer at pH 9.5. After each incubation plates were washed four times with 0.05% (v/v) Tween 20 in demi water in order to remove unbound proteins. The wells were then successively incubated 1 hr at ambient temperature with 100 Fl antibody solution in blocking buffer (PBS containing 2% (w/v) BSA; 0.05% (v/v) Tween 20; 1% (v/v) culture supernatant of a *S. cerevisiae* strain, SU 50 (Giuseppin, et al., 1993) grown on YPD; 1% (v/v) of a cleared lysate of *E. coli* JM109 cells). The antigen sensitized plates were then successively incubated with (1) serially diluted llama serum samples, (2) 2000-fold diluted polyclonal rabbit anti llama serum (obtained via immunizing rabbits with llama immunoglobulines which were purified via ProtA and ProtG

columns), (3) 2000-fold diluted swine-anti-rabbit immunoglobulins conjugated with horse radish peroxidase. The bound peroxidase activity was determined using the substrate 3,3',5,5'-tetramethylbenzidine.

5

**Example II.2 - Cloning, expressing and screening of llama V<sub>HH</sub> fragments.**

**II.2.1 Isolation of gene fragments encoding llama V<sub>HH</sub>**

10 

**domains.**

From an immunized llama in accordance to Example II a blood sample of about 200 ml was taken and an enriched lymphocyte population was obtained via Ficoll (Pharmacia) discontinuous gradient centrifugation. From these cells, total RNA was isolated by acid guanidium thiocyanate extraction (e.g. via the method described by Chomczynski and Sacchi (1987). After first strand cDNA synthesis (e.g. with the Amersham first strand cDNA kit), DNA fragments encoding V<sub>HH</sub> fragments and part of the long or short hinge region were amplified by PCR using specific primers:

PstI

V<sub>H</sub> - 2B      5'-AGGTSMARCTGCAGSAGTCWGG-3'      (see SEQ. ID. NO: 1)

S = C and G, M = A and C, R = A and G, W = A and T,

25

HindIII

Lam-075'-AACAGTTAAGCTTCCGCTTGCGCCGCGGAGCTGGGGTCTTCGCTGTGGTGCG-3'

(short hinge)

(see SEQ. ID. NO: 2)

30

HindIII

Lam-085'-AACAGTTAAGCTTCCGCTTGCGCCGCTGGTTGTGGTTTTGGTGTCTTGGGTT-3'

(long hinge)

(see SEQ. ID. NO: 3)

Upon digestion of the PCR fragments with *Pst*I (coinciding with codon 4 and 5 of the  $V_{HH}$  domain, encoding the amino acids L-Q) and *Bst*EII (located at the 3'-end of the  $V_{HH}$  gene fragments, outside and upstream of the 08 5 primer coinciding with the amino acid sequence Q-V-T), the DNA fragments with a length between 300 and 450bp (encoding the  $V_{HH}$  domain, but lacking the first three and the last three codons) were purified via gel electrophoresis and isolation from the agarose gel.

10

#### II.2.2 Construction of *Saccharomyces cerevisiae* expression plasmids encoding llama $V_{HH}$ domains

Plasmids pUR4547 (CBS 100012) and pUR4548 (CBS 15 100013) are *Saccharomyces cerevisiae* episomal expression plasmids, derived from pSY1 (Harmsen et al., 1993). Both plasmids contain the GAL7 promoter and PGK terminator sequences as well as the invertase (SUC2) signal sequence. In both plasmids the DNA sequence encoding the SUC2 signal 20 sequence is followed by the first 5 codons (encoding Q-V-Q-L-Q; see SEQ. ID. NO: 4) of the  $V_{HH}$  domain (including the *Bst*II site), a stuffer sequence, the last six codons (encoding Q-V-T-V-S-S; see SEQ. ID. NO: 5) of the  $V_{HH}$  domain. In pUR4547, this is followed by two stop codons, an 25 *Afl*III and *Hind*III site. In pUR4548, the Q-V-T-V-S-S (SEQ. ID. No: 5) sequence is followed by eleven codons encoding the myc-tag, two stop codons, an *Afl*III and *Hind*III site.

Plasmids pUR4547 and pUR4548 were deposited under 30 the Budapest Treaty at the Centraal Bureau voor Schimmelcultures, Baarn (The Netherlands) on 18th August 1997 with deposition numbers CBS 100012 and CBS 100013,

respectively. In accordance with Rule 28(4) EPC, or a similar arrangement from a state not being a contracting state of the EPC, it is hereby requested that a sample of such deposit, when requested, will be submitted to an expert only.

Upon digesting pUR4548 with *Pst*I and *Bst*EII, the about 6.4kb vector fragment was isolated and ligated with the *Pst*I-*Bst*EII fragments of between 300 and 450 bp obtained as described above. After transformation of *S. cerevisiae* via electroporation, transformants were selected from minimal medium agar plates (comprising 0.7% yeast nitrogen base, 2% glucose and 2% agar, supplemented with the essential amino acids and bases).

15

### II.2.3 Screening for antigen specific V<sub>HH</sub> domains

For the production of llama V<sub>HH</sub> fragments with myc-tail, individual transformants were grown overnight in selective minimal medium (comprising 0.7% yeast nitrogen base, 2% glucose, supplemented with the essential amino acids and bases) and subsequently diluted ten times in YPGal medium (comprising 1% yeast extract, 2% bacto pepton and 5% galactose). After 24 and 48 hours of growth, the culture supernatant of the colonies was analysed by ELISA for the presence of V<sub>HH</sub> fragments which specifically bind to the *E. coli* K88 antigen, in essential the same way as described in Example 1. In this case, however, the presence of specifically bound V<sub>HH</sub> fragments was detected by incubation with monoclonal anti-myc antibodies, followed by incubation with polyclonal rabbit-anti-mouse conjugate with alkaline phosphatase.



In this way a number of  $V_{HH}$  fragments have been obtained, which specifically recognise the *E. coli* K88 antigen.

5

Two examples of such fragments are given below:

K607  
 QVQLQESGGG LVQPGGSLRL SCAASGSIFS ASAMTWYRQA PGKSREYVAR IFFSGGTNYA  
 DSVKGRFTIS RDNAKNTMYL QMNDLKREDT AVYYCNLLSY WGQGTQVTVS S  
 10 (see SEQ. ID. NO: 6)

K609  
 QVQLQESGGG LVQPGGSLRL SCAASGGTFS WYAMGWFRQA PGKEREVAT VSRGGGSTYY  
 ADSVKGRFTI SRDNAKNTVY LQMNSLKPED TAAYYCAAGR GSPSDTGRPD EYDWGQGTQ  
 15 VTVSS  
 (see SEQ. ID. NO: 7)

### Example II.3 - Construction of "self-cloning" yeast

20 expression cassette, encoding a chimeric protein anchored to the cell wall of yeast, comprising a binding domain and a cell wall anchor

In order to display a binding domain at the yeast  
 25 cell wall, a genetic fusion of the gene encoding the binding domain and a gene encoding the cell wall anchor has to be constructed, either with or without a linker sequence, essentially as described in WO 94/25591. As an example, the construction of a "self cloned" yeast,  
 30 displaying a llama  $V_{HH}$  fragment fused via the "long hinge" region to the  $\alpha$ -agglutinin cell wall anchor will be described below. Obviously this can be done in much the same way for different binding domains, different linkers/hinges and different cell wall anchors.

35

### II.3.1 Isolation of the llama "heavy chain" long hinge sequence.

For the isolation of the hinge regions of the llama  
 5 "heavy chain" antibodies, the cDNA which was obtained as  
 described in Example 1, was amplified by PCR using the  
 primers V<sub>H</sub>-2B and Lam-03. The nucleotide sequence of the  
 latter primer was based on a consensus sequence based on  
 the C<sub>H</sub>2 domains of different species. The PCR reaction.  
 10 resulted in three DNA fragments of about 450 (a), 550 (b)  
 and 850 (c) bp.

Lam-03 BamHI  
 15 GGTATGGATCCACRTCCACCACCACRCAYGTGACCT  
 (see SEQ. ID. NO: 8)

Upon digesting these fragments with *Pst*I and *Bam*HI  
 and size fractionation, they were ligated into pEMBL  
 20 (Dente, 1983) and subjected to nucleotide sequence  
 analysis. The clones obtained from the DNA fragment with  
 an average length of ~450 bp (a) were found to comprise a  
 short hinge region, having the following sequence:

25 V<sub>HH</sub> >< short hinge >  
 GGTCACCGTCTCCTCAGCGCACCAAGACCCAGCTCCAAGTGTCCCAAATGCCCA  
 V T V S S A H H S E D P S S K C P K C P  
 <- LAM 07 WB <-1  
 (see SEQ. ID. NO: 9)  
 30

The clones obtained from the DNA fragment with an  
 average length of ~550 bp (b) was found to comprise a long  
 hinge region, having the following sequence:

35 BstEII V<sub>HH</sub> >< long hinge  
 GGTCACCGTCTCCTCAGAACCCAAGACACCAAAACCACAACCACAACCACAACCACAACCACAAT  
 V T V S S E P K T P K P Q P Q P Q P Q P Q P N

&lt;- LAM 08 WB &lt;-1

>

CCTACAAYAGAATCCAAGTGTCCCAAGTGTCCA  
 5 P T T E S K C P K C P

(see SEQ. ID. NO: 10)

For the construction of pUR4588 (Fig 5), a *NheI* site was introduced in the 3'-end of the long hinge region via PCR using the primers:

*NheI*

BOLI 18 CGCGGCTAGCCTTGGATTCTGTTGTAGGATTGGGTTG  
 15 (see SEQ. ID. NO: 11)

*BstEII*

LH CCCAGGTCACCGTCTCCTCAGAACCCAAG  
 20 via which the following sequence: (see SEQ. ID. NO. 12)

CCTACAACAGAATCCAAGTGTCCCAAGTGTCCA  
 P T T E S K C P K C P  
 25 became (see SEQ. ID. NO: 13)

*NheI*

CCTACAACAGAATCCAAGGCTAGC  
 P T T E S K A S  
 30 (see SEQ. ID. NO: 14)

Upon digestion with *BstEII* and *NheI*, an about 90 bp fragment was obtained encoding the last 4 amino acids of the  $V_{HH}$  domain and the long hinge region except for the last 5 amino acids.

### II.3.2 Adaptation of the $\alpha$ -agglutinin gene

Plasmid pUR4482 (fig 4) is a yeast episomal  
5 expression plasmid for expression of a fusion protein with  
the invertase signal sequence, the CH<sub>v</sub>09 variable region,  
the Myc-tail and the Camel "X-P-X-P" Hinge region (see  
Hamers-Casterman et al., 1993), and the  $\alpha$ -agglutinin cell  
wall anchor region.

10

The *Pst*I-site at position 1665 in the  $\alpha$ -agglutinin  
gene was removed via a three step PCR using primers PAF01  
to PAF04. For easy cloning and sequencing in pEMBL8, *Eco*RI  
and *Hind*III sites were introduced at the 3'- and 5'-end of  
15 the gene fragment encoding  $\alpha$ -agglutinin, respectively. PCR-  
A using primers PAF01 and PAF02 on a pUR4482 template  
resulted in an about 707 bp fragment with the *Eco*RI  
restriction site at the 3'-end. PCR-B using primers PAF03  
and PAF04 on a pUR4482 template resulted in an about 427 bp  
20 fragment with the *Hind*III site at the 5'-end. A third PCR  
was performed with the fragments obtained from reaction A  
and B, together with the primers PAF01 and PAF04. After  
digesting the obtained PCR product (~1135 bp), which  
comprises the modified  $\alpha$ -agglutinin gene without the *Pst*I  
25 restriction site, with *Eco*RI and *Hind*III, the resulting  
~1130 bp fragment was ligated into the vector fragment of  
pEMBL8 which was digested with the same enzymes resulting  
in pRL03.

## PCR-A

PAF01 GGAATTCGTCTCCTCAGAACAAAAAC (see SEQ. ID. NO: 15)  
 PAF02 GCTGCTGCAAAAGGAATTTA (see SEQ. ID. NO: 16)

## 5 PCR-B

PAF03 AAATTCCTTTTGCAGCAGC (see SEQ. ID. NO: 17)  
 PAF04 GGGAAGCTTCGACAAAAGCAGAAAAATGA (see SEQ. ID. NO: 18)

In essentially the same way as for the removal of  
 10 the *Pst*I site, the *Sap*I site in the  $\alpha$ -agglutinin coding  
 sequence at position 1618 was removed from plasmid pRL03.  
 PAF01 and BOLI-20 were used in a PCR reaction with pRL03 as  
 template to generate an approximately 660 bp fragment and  
 primers BOLI19 and PAF04 were used to make an approximately  
 15 504 bp fragment which were linked by splicing by overlap  
 extension using primers PAF01 and PAF04 (PCR-C).

## PCR-A

PAF01 see above  
 20 BOLI-20 TTACAAAAGTGGGTTCTTCAGATGGAA (see SEQ. ID. NO: 19)

## PCR-B

BOLI-19 TTCCATCTGAAGAACCCACTTTTGTA (see SEQ. ID. NO: 20)  
 PAF04 see above

25

## PCR-C

Product of PCR-A and PCR-B and PAF01 and PAF04.

30 Again an about 1130 bp *Eco*RI-*Hind*III fragment was  
 obtained and cloned in pEMBL8.

Finally, the *Sac*I site at position 2041 can be  
 removed and a *Hind*III site can be introduced downstream of  
 35 the stop codon by replacing the sequence:

SacI HindIII  
 gagctCGGTTCGATCATTCTGCTTTTGTGCGAagctt  
 cTCGAGCCAAGCTAGTAAAAAGACGAAAACAGCTTCGAa  
 E L G S I I F L L L S

5

with a synthetic linker having the sequence:

(SacI) HindIII  
 gagctGGGTTCGATCATTCTGCTTTTGTGCTACCTGCTATTCTAAGATCTGATTAAACGCGTGAagctt  
 10 cTCGACCCAAGCTAGTAAAAAGACGAAAACAGCATGGACGATAAGATTCTAGACTAATTTCGCACTTCGAa  
 E L G S I I F L L L S Y L L F \*  
 (see SEQ. ID. NO: 21)

In this way a plasmid was obtained containing an  
 15 about 990 bp *NheI*-*HindIII* fragment encoding the C-terminal  
 320 amino acids of the  $\alpha$ -agglutinin in which the *PstI*, *SapI*  
 and *SacI* sites were removed via silent mutations in order  
 to facilitate further construction work.

### 20 II.3.3 Construction of pUR4588

The following fragments were subsequently joined:

i) the about 100 bp *SacI* - *PstI* fragment of pUR4548:  
 comprising a part of the *Gal7* promoter, the *SUC2* nucleotide  
 25 sequence and the first 4 codons of the  $V_{HH}$  fragment,

ii) the about 350 bp *PstI* - *BstEII* fragment, which was  
obtained as described in Example 2: encoding a truncated  
 $V_{HH}$  fragment, missing both the first 4 (QVQL; see SEQ. ID.  
 30 NO: 4) and the last 5 (VTVSS; see SEQ. ID. NO: 5) amino  
 acids of the  $V_{HH}$  fragment,

iii) the about 90 bp *BstEII* - *NheI* fragment obtained as  
described in Example 3.1: encoding the last 5 amino acids  
 35 of the  $V_{HH}$  fragment and the long hinge region, and

iv) the about 1 kb *NheI* - *HindIII* fragment obtained as described in 3.2: the adapted  $\alpha$ -agglutinin gene.

Finally, the thus obtained about 1.5 kb *SacI*-  
5 *HindIII* fragment was ligated into the about 7.5 kb vector fragment of pUR2822 which was digested with the same enzymes, resulting in pUR4588 (Figure 7).

Plasmid pUR2822 was deposited under the Budapest  
10 Treaty at the Centraal Bureau voor Schimmelcultures, Baarn (The Netherlands) on 29 September 1998 with deposition number CBS 101292. In accordance with Rule 28(4) EPC, or a similar arrangement from a state not being a contracting state of the EPC, it is hereby requested that a sample of  
15 such deposit, when requested, will be submitted to an expert only.

#### II.3.4 Production of yeast strains "functionally" expressing the chimeric protein

20

Plasmid pUR4588 (fig5) was digested with *SapI*, after which an about 6.7 kb fragment was purified, via gel electrophoresis and isolated from the agarose gel. This fragment was introduced into yeast strains via  
25 electroporation. Transformants containing (multicopies of) the DNA fragment integrated into the rDNA locus of their genome were selected as described in Example 2. Induction of the production and display of the binding domain was also done as described in Example 2. In order to determine  
30 the functionality of the binding domains displayed at the yeast cell wall, the following assay was performed:

The yeast cells were diluted in PBS containing 0.05% (v/v) Tween 20 (PBST) to an  $A_{660} = 1$  and 10  $\mu$ l of this suspension was mixed with 1  $\mu$ g K88ac antigen in 100  $\mu$ l PBST 5 in an Eppendorf tube and incubated for 1 hr at ambient temperature. Unbound antigen was removed from the yeast cells by three washes in 1 ml PBST. Cells were collected after each wash by centrifugation and aspiration of the supernatant. The yeast cells were subsequently resuspended 10 in 100  $\mu$ l of PBST containing a conjugate of monoclonal antibody AD11, which is specific for K88, and horse radish peroxidase (AD11-HRPO; Van Zijderveld et al., 1990). After 1 hr incubation at ambient temperature the cells were washed again three times with PBST. The amount of AD11-HRPO 15 bound to the yeast cells was taken as a measure of the amount of functional K88-binding domains displayed at the yeast cell wall. This value was determined by performing a peroxidase assay on serial twofold dilutions of these cells, using 3,3',5,5'-tetramethylbenzidine as a substrate, 20 and measuring the  $A_{450}$ . The recovered functionality (RF) of the displayed antibody fragments after a physical treatment to inactivate the *S. cerevisiae* cells is defined as the  $A_{450}$  before treatment (is F0) divided by the  $A_{450}$  after treatment (is Ft).



**Example III- Heat inactivation of  $V_{HH}$ s****5 III.1 Production of biomass suspension.**

*Saccharomyces cerevisiae* strain SU50 containing the genomically integrated expression plasmid pUR4588 (Fig5) was used. The strain was grown in a 10 l scale fermentor using a 10 fed batch fermentation set up (Mendoza-Vega et al., 1994). Such a fermentation consists of two steps: the first step (the batch phase) is performed in a Yeast extract and Peptone (YP) medium containing glucose. In the second step (the feed phase) YP medium is added containing glucose and 15 galactose, in order to induce the promoter for the production of the  $V_{HH}$ -agglutinine fusion. In this way a culture was obtained with a cell density of about  $10^9$ - $10^{10}$  per ml, comparable to large scale fermentations.

20  $N$ , Cell density or viable counts ( $N$ ) were determined by making decimal dilutions of a cell culture in sterile pepton physiological salt (PFS; 0.85% NaCl, 0.1% Bactopepton (Difco), pH 7). Samples of these dilutions were plated on YPD agar and incubated for 5 days at 30 °C after which the 25 number of colonies were counted. The reduction of viable counts (RVC) after treatment to inactivate the *S. cerevisiae* cells is defined as the logarithm of the viable counts after treatment (is  $N_t$ ) divided by the number of viable counts before treatment (is  $N_0$ );  $\text{Log } (N_t/N_0)$ .

### III.2 Heat treatment

Determination of the killing efficiency of heat treatment on high cell density cultures was performed using one of two different approaches, as desired.

#### III.2.1 Using glass capillaries

Sterile glass capillaries (Fisher Scientific Den Bosch, l=100 mm, d=2 mm) were filled with 0.1 ml of the yeast culture medium and sealed. After incubating the filled, sealed capillaries, they were placed for a set time in a water bath at the desired temperature (experiments were performed at quadruplets). After this the recovered functionality (RF) and the reduction in viable counts (RVC) for the different samples was determined as described above.

#### III.2.2 Using aluminium vials

Sterile aluminium cryo vials (Omnilabo, volume 3 ml) were filled with 0.5 ml of the yeast culture for each temperature/time combination (experiments performed in duplo). The vials were placed in a water bath at the desired temperature and time. After this the recovered functionality (RF) and the reduction in viable counts (RVC) for the different samples was determined as described above.

### III.3 Viable counts and GI-ELISA

From the yeast culture the viable counts (NO) and

the GI-ELISA signal ( $F_0$ ) was determined before heat treatment. The same was done for the samples obtained as described above ( $N_t$  and  $F_t$ , respectively). The results are presented in Table 3 below.

5 Table 3

Temp (°C)	time (sec)	RVC (= log ( $N_t/N_0$ ))	RF (= $F_t/F_0$ )
60	300	- 4	100%
62	60	> -6	100%
70	30	> -7	> 85%
74	15	> -7	> 85%

**Example IV - Inactivation of *S. cerevisiae* SU50 (pUR 4588) with Ultra High Pressure**

5

From literature it is known that at pressures over 300 MPa proteins FF ATP-ase can be inactivated (Wouters et al, 1998).

10

*S. cerevisiae* SU50 (pUR4588), displaying anti-*E. coli* K88<sub>V<sub>HH</sub></sub> fragments, was grown at 10 liter scale as described above. Samples of 1.5 ml were taken and transferred to bags for Ultra High Pressure treatment in a "Food Lab Multi Vessel" (Stansted Fluid Power, UK). Samples 15 were incubated at three different pressures 250, 300 and 325 Mpa for different periods of time. The survival of the yeast cells and the functionality of the displayed V<sub>HH</sub>s was determined as described above. The results are presented in Table 4 below.

20

Table 4

Pressure (Mpa)	time (min)	RVC (=log (Nt/N0))	RF (=Ft/F0)
250	60	- 9	100%
300	3	- 6	>70%
	20	- 9	>70%
325	1	- 6	70%
	3	- 9	70%
	20	- 9	70%

25

It was further found that yeast cells that do not have the capacity to synthesize trehalose are much more

sensitive for UHP (Fernandes et al., 1997). Therefore, a preferred embodiment of the inactivation of yeast with UHP is, instead of using a wild type yeast strain, using a variant strain defective in the synthesis of trehalose, 5 e.g. a strain in which the TPS1 gene has been inactivated e.g. by partial or complete deletion. In this way the UHP treatment could be reduced from 300 MPa to 200 MPa with the same reduction in viable counts.

**Example V- Killing of yeast cells by irradiation**

5           An alternative for the inactivation of yeast cells is gamma radiation. *S. cerevisiae* SU50 (pUR4588), was grown at 10 liter scale as described above. After fermentation the biomass slurry was transferred to polypropylene drums and exposed to 10 kGray or 25 kGray of gamma radiation  
10 ('Gammaster', Ede). The survival of the yeast cells and the functionality of the displayed  $V_{HHS}$  was determined as described above. For both cases, the RVC was better than - 10 (no viable counts left after irradiation), whereas the RF of the  $V_{HHS}$  was 100%. The anti-K88 activity was not  
15 affected by the irradiation.

          A number of genes involved in DNA repair are known (e.g *rad9*, *rad30*, *rad52*, *sir2*, *sir3*, *sir4*). Yeast strains carrying mutations in these genes, are known to be more  
20 sensitive towards radiation (Friedberg et al., 1991; Tsukamoto et al., 1997). In a preferred embodiment of this invention, a yeast strain with one or more mutations in one or more of these genes is used.

**Example VI - Combined effect of heat inactivation and antimicrobials on *Saccharomyces cerevisiae***

5            *S. cerevisiae* SU50 (pUR4588) cells were cultivated and harvested as described above and subjected to heat treatment as described above in the presence of antimicrobials like ethanol, potassium sorbate and antimicrobial peptides like nisin and MB21. According to  
10 the methods described in the previous examples the inactivation efficiency and the recovery of the functionality of the  $V_{HH}$  antibodies have been determined. The results are given in Table 5 below.

15 Table 5

Inactivation of *S. cerevisiae* and  $V_{HH}$  as function of the temperature and the concentration of antimicrobials

Temp (°C)	Antimicrobials	Time (min)	RVC (=log $N_t/N_0$ )	RF (= $F_t/F_0$ )
50	3% ethanol	60	0	100%
50	0.2% K-sorbate	60	0	100%
60	---	5	-4	100%
60	3% ethanol	5	-7	90%
60	0.2% K- sorbate	5	-8	95%

20

It has been reported that yeast cells that do not have the full capacity of synthesising multi drug resistant proteins are much more sensitive to weak acids, e. g. sorbic acid (PDR12; Piper et al., 1998). Therefore, a  
25 preferred embodiment of inactivation of yeast by a

combination of heat and acid is to use, instead of the wild type yeast strain, a strain in which the PMR gene is inactivated, e.g. via partial or complete deletion. In this way either the temperature or the amount of acid or both 5 can be reduced considerably.



**Example VII - Animal feeding trials with yeast displaying anti *E. coli* K88 V<sub>HH</sub>s on their surface**

5           In this experiment two groups of piglets were used. A mild *E. coli* K88 infection was induced in all animals by an oral dose of  $10^6$  *E. coli* K88 cells. For group I (exp. group) the feed was supplemented with 6 ml of a concentrated suspension ( $10^{10}$ - $10^{11}$  cells per ml) of SU50  
10 (pUR4588), and for group II (control) with the same amount of wild type SU50 yeast. The yeast cells were inactivated with gamma irradiation according to the procedure as described in Example 6.

15           Number of affected piglets due to dosing of  $>10^6$  *E. coli* K88/g in the meal.  
See Table 6 below.

Table 6

20

Day	Control	Exp group
0	0	0
1	22	17
2	50	19

Conclusion: protective effect clearly visible after 2 days.

**Example VIII Dye Transfer Prevention using HCV antibodies.**

Cotton swatches dyed with RR6 were prepared by soaking pieces of cotton (5\*5 cm) in a solution of RR6 for 30 min (0.1 M borate buffer, pH 8.5, 0.15 M NaCl). After drying, these swatches were washed in the presence of various concentrations of a VHH (R7 anti-RR6 as disclosed in example 1). For the monitoring of dye transfer, a white cotton swatch (dye pick-up swatch) was added. As wash solution, a carbonate buffer (pH 9) was used, to which in a number of experiments surfactants were added. These were alkyl benzene sulfonate (LAS, 600 mg/l), and a LAS/Synperonic A7 mixture (290 mg/l LAS, 660 mg/l SA7).

After the wash the swatches were line dried and the reflectance spectra were measured using a Minolta spectrometer. The data thereby obtained were transferred to the CIELAB  $L^*a^*b^*$  colour space parameters. In this colour space,  $L^*$  indicates lightness and  $a^*$  and  $b^*$  are the chromaticity coordinates. The colour differences between the swatches prior to washing and after the wash, were expressed as  $\Delta E$  indicating the distance in the colour space between before and after washing.

The results, given as the  $\Delta L$ ,  $\Delta a$ ,  $\Delta b$ , and  $\Delta E$  of the dye pick-up swatches after the experiment, with respect to white cotton are given in the table below.

Table 7

Conditions applied	$\Delta L$	$\Delta a$	$\Delta b$	$\Delta E$
Buffer, pH 7 containing VHH against RR6 at concentration:				
0 $\mu M$	2.9	-5.7	2.2	6.8
1.25 $\mu M$	1.9	-4.1	1.7	4.9
2.5 $\mu M$	2.0	-4.3	1.9	5.2
5.0 $\mu M$	0.6	-1.4	0.8	1.8
10.0 $\mu M$	0.6	-1.1	0.6	1.4
Buffer, pH 9 containing VHH against RR6 at concentration:				
0 $\mu M$	3.3	-5.4	2.1	6.7
1.25 $\mu M$	2.1	-4.8	2.0	5.7
2.5 $\mu M$	1.6	-3.5	1.5	4.2
5.0 $\mu M$	0.7	-1.7	0.8	2.1
10.0 $\mu M$	0.1	-0.5	0.4	0.7
LAS solution, pH 9, containing VHH against RR6 at concentration:				
0 $\mu M$	2.0	-4.3	1.9	5.1
10.0 $\mu M$	1.1	-2.6	1.2	3.1
LAS/SA7 solution, pH 9, containing VHH against RR6 at concentration:				
0 $\mu M$	2.0	-4.2	1.8	5.0
10.0 $\mu M$	0.1	-0.2	0.1	0.3

5        These results illustrate that anti-RR6 antibodies are  
able to prevent dye transfer, especially at levels of above  
2.5  $\mu M$  during use and that these VHHs remain stable in the  
presence of surfactants especially mixed surfactants and  
can hence continue to be effective during the use of  
10 laundry detergents.

**EXAMPLE IX Induction of a humoral immune response in llama.**

A male llama was immunised with an oil emulsion (1:9 V/V, antigen in PBS : Specol (Bokhout et al.) subcutaneously 5 and intramuscularly. Per site about 0.75-1.5 ml emulsion was injected containing about 100 µg of a conjugate consisting of the azo-dye RR120 (Cibacron Red 4G-E, Ciba Geigy) which was coupled to BSA via its reactive triazine group.

10 Immunisations were performed according to the following time schedule: the second immunisation was performed three weeks after the first injection, and the third immunisation two weeks after the second one. The immune response against RR120 was followed by titration of  
15 serum samples in ELISA by using Nunc Covalink plates, which were coated with the azo-dye-RR120 (100 µl/well of 2.5 mM RR120 in coating buffer (0.1 M di-sodium tetraborate decahydrate, 0.15 M NaCl pH 8.5), incubation overnight at 50°C).

20

After incubation with serum, the bound llama antibodies were detected with poly-clonal rabbit-anti-llama antiserum (obtained via immunising rabbits with llama immunoglobulines purified via ProtA and ProtG columns; ID-  
25 DLO) and, as a second step, swine-anti-rabbit immunoglobulines (Dako) conjugated with alkaline phosphatase were used after which the alkaline phosphatase enzyme-activity was determined upon incubation with p-nitro-phenyl phosphate and the optical density was measured at 405nm.

30

**EXAMPLE IX.2. Cloning, selection and screening of llama V<sub>HH</sub> fragments**

5 IX.2.1 Isolation of gene fragments encoding llama V<sub>HH</sub> domains.

From an immunised llama a blood sample of about 200 ml was taken and an enriched lymphocyte population was obtained  
10 via centrifugation on a Ficoll (Pharmacia) discontinuous gradient. From these cells, total RNA was isolated by acid guanidium thiocyanate extraction (e.g. via the method described by Chomczynski and Sacchi, 1987). After first strand cDNA synthesis using MMLV-RT (Gibco-BRL) and random  
15 oligonucleotide primers (Pharmacia), or by using the Amersham first strand cDNA kit, DNA fragments encoding V<sub>HH</sub> fragments and part of the long or short hinge region were amplified by PCR using specific primers of SEQ ID 1, SEQ ID 2 and SEQ ID 3 as described above.

20

IX.2.2 Construction and screening of *S. cerevisiae* libraries which secrete V<sub>HH</sub> domains in the culture medium.

The DNA-fragments generated by PCR were digested with  
25 *Pst*I (coinciding with codon 4 and 5 of the V<sub>HH</sub> domain, encoding the amino acids L-Q) and *Bst*EII (naturally occurring in FR4 region of the majority of the V<sub>HH</sub>-genes and coinciding with the amino acid sequence Q-V-T). The digested PCR-products, with a length between 300 and 400bp (encoding  
30 the complete V<sub>HH</sub> domain, but lacking the first and the last three codons), were separated by electrophoresis on agarosegel and, after purification from the gel-slices with

the Qiaex-II extraction kit, cloned in the *Saccharomyces cerevisiae* episomal expression plasmids pUR4547 or pUR4548

RR120 specific  $V_{HH}$  fragments were isolated via  
5 screening of culture supernatants from individual clones in ELISA using either

- rabbit anti-llama  $V_{HH}$  poly-clonal antibody followed by incubation with goat anti-rabbit poly-clonal antibodies  
10 conjugated to horse radish peroxidase (Bio-rad) (for  $V_{HH}$  fragments cloned in pUR4547); or
- mouse anti-myc mono-clonal antibody 9E10 followed by incubation with poly-clonal goat-anti-mouse conjugate  
15 with horse radish peroxidase (for  $V_{HH}$  genes cloned in pUR4548) for detection.

For the production of llama  $V_{HH}$  fragments in *S. cerevisiae*, individual transformants were grown overnight in  
20 selective minimal medium (comprising 0.7% yeast nitrogen base, 2% glucose, supplemented with the essential amino acids and bases) and subsequently diluted ten times in YPGal medium (comprising 1% yeast extract, 2% bacto pepton and 5% galactose). After 24 and 48 hours of growth, the culture  
25 supernatant of the colonies was analysed by ELISA for the presence of antigen specific  $V_{HH}$  fragments.

IX.2.3 Construction and screening of *S. cerevisiae* libraries which display VHH domains at the cell surface.

The PCR-products obtained as described in IX.2.1 can be  
5 digested with *Pst*I and *Bst*EII and cloned in the yeast  
display vector pUR4659 (fig7) as gene-fragments encoding  
the V<sub>HH</sub> -domain including the hinge region. In this way V<sub>HH</sub>  
antibody domains were displayed on yeast cell surface by  
means of N-terminal fusions onto the cell wall protein  $\alpha$ -  
10 agglutinin (see patent WO 94/01567). Such a yeast display  
library with  $1 \times 10^6$  clones was constructed in the episomal  
yeast expression vector pUR4659. Transformed yeast cells  
were pooled and grown for about 17 hours on selective  
minimal medium until the OD<sub>660</sub> was 2. Subsequently, these  
15 cells were inoculated into YPGal for expression, secretion  
and display of the V<sub>HH</sub> antibody fragments on the cell  
surface and grown for appr. 17 hours. Secreted V<sub>HH</sub> -  
fragments were removed by washing the cells with PBS,  
thereby avoiding a disturbing competition for binding to  
20 antigen between yeast bound and free V<sub>HH</sub> domains.

For the isolation of RR120 binding VHH fragments, the  
VHH displaying *S. cerevisiae* cells were incubated for two  
hours with *in vitro* biotinylated BSA-RR120 in PBS followed  
25 by an incubation with FITC-labelled streptavidin.

Fluorescence of individual yeast cells was analysed by means  
of a FACS (Fluorescence Activated Cell Sorter) and the cells  
with give above average fluorescent signals were selected  
and inoculated in minimal medium for a second round  
30 selection. In this second round, cells with the highest  
fluorescent signals were selected and propagated as single

colonies for analysis or grown for further rounds of selections.

Individual *S. cerevisiae* clones were grown on YPgal  
5 for 48 hours in wells of microtiter plates. During the  
second part of this incubation, fusion protein consisting of  
a V<sub>HH</sub> fragment and  $\alpha$ -agglutinin is secreted in the growth-  
medium. Culture supernatants containing these chimeric  
molecules were tested in ELISA for binding to RR120 using  
10 the myc-TAG for detection as described above. For further  
analysis of selected clones, V<sub>HH</sub>-encoding DNA fragments from  
these yeast display vectors were recloned in the episomal  
yeast secretion vector pUR4548 and analysed as described in  
paragraph 2.2.

15

IX. 2.4 Construction and screening of a phage library  
displaying VHH domains

Figure 6 gives a schematic representation of the phagemid  
20 vector pUR4676 which was assembled from:

- the about 2.85 kbp *NcoI*/*ScaI* fragment of pFab5c(Ørum et al., 1993). The *BstEII* site present in the *lacI* gene was removed via a silent mutation using PCR mutagenesis.

25

- the about 2.7 kbp *NcoI*/*ScaI* fragment of pHEN1 (Hoogeboom et al., 1991).

The DNA fragment between the *PstI* site and the first codons  
30 of geneIII were replaced with the following sequence:



```

5  PstI          BstBII          NotI          HindIII
   <- MPE 28 WB (22)  -><- MPE 42 WB (26)  -><- MPE 44 WB (25)
   ctgcaGGAGTCATAATGAGGGACCCAGGTCACCGTCTCCTCAGCGGCCGCAAGCGGAAGCTTAGAACAAA
10  gACGTCCTCAGTATTACTCCCTGGGTCCAGTGGCAGAGGAGTCGCCGGCGTTCGCCTTCGAATCTTGTTT
   <- MPE 27 WB (36)  -><- MPE 41 WB (26)  -><-
   [-> cassette HC-V [-] [->

15  (HindIII)
   -><- MPE 32 WB (30)  -><- MPE 46 WB (27)  ->
   AACTCATCTCAGAAGAGGATCTGAATGGGGCCGCACATCACCATCATCACCATGGGGCCGCATAGagcttAACTGTTGAA
   TTGAGTAGAGTCTTCTCCTAGACTTACCCCGGCGTGTAGTGGTAGTGGTACCCCGGCGTATCTCGAaTTGACAACTT
20  MPE 43 (25)  -><- MPE 31 WB (30)  -><- MPE 45 WB (21)  ->
   myc tail [-] [->His (6) tail<-] amber [-> G III

```

resulting in pUR4676.

Upon digesting this vector with *Pst*I and *Hind*III and subsequent ligation in the presence of the PCR fragments produced as described in 2.1 and digested with *Pst*I / *Hind*III, the VHH phagemid libraries were obtained.

#### IX.2.2.1. Selection of the VHH library.

From the phagemid libraries, VHH displaying phage particulates were produced via superinfection with phage M13-K07 (Promega) essentially as described by (Marks et al., 1991). Selection of the VHH phage library was performed essentially as described by (Marks et al., 1991) via three rounds of panning using RR120 coated immunotubes. The immunotubes (Nunc; Maxisorp, 75 x 12 mm) were coated with 4 ml 2.5 mM RR120 in coating buffer (0.1 M di-sodium tetraborate decahydrate and 0.15 M NaCl pH 8.5) overnight at 50°C. After blocking with Marvel (2% in PBS) and washing

with PBS, about  $10^{12}$  phages (preincubated in 4 ml 2% Marvel in PBS for 15 minutes) were added to the tubes. Upon incubation for 1 hour (on turntable) the tubes were washed 15 times with PBST (PBS with 0.1% tween) and 2 times with 5 PBS. Bound phages were subsequently eluted by adding 2 ml 100 mM triethylamine.

The eluted phages were added to 1 M Tris pH 7.4 for neutralisation before adding *E. coli* cells for 10 infection/phage rescue. This process was repeated three times.

Subsequently, single *E. coli* colonies were transferred to microtiter plates and grown overnight in 150 microliter 15 2x TY medium containing ampicillin (100 microgram per ml) and IPTG (0.8 mM). The culture supernatant was subsequently tested in an antigen specific ELISA.

In this way a large number of RR120 binding VHH 20 domains could be isolated.

For further analysis of selected clones,  $V_{HH}$ -encoding DNA fragments from these phage display vectors can be recloned in the episomal yeast secretion vector pUR4548 and 25 analysed as described in paragraph IX.2.2.

### IX.3 Selection of RR120 recognising $V_{HH}$ domains via yeast display in consumer products under application conditions.

30 To enrich for VHH fragments stable in product formulations the screening as described in 2.3 was repeated using application conditions.

In this example we have dissolved the yeast cells in commercially available detergent formulations resp 6 g/l Dutch Omo® (without protease, hereafter referred to as Omo) followed by the addition of the biotinylated BSA-RR120. After a one hour incubation under application conditions, unbound RR120 and detergent were discarded by centrifugation. Subsequently, the cells were incubated with FITC-labelled streptavidin and analysed as described above.

10

IX.4 Selection of RR120 recognising  $V_{HH}$  domains via phage display in consumer products under application conditions.

IX.4.1 Initial selection

15 Selection of phages binding to the RR120 antigen under application conditions was essentially performed as described in example 2.4, but using the following conditions during the selection of the phages in the immunotubes:

- 20 1. 0.6 g/l OMO-MA in 25 mM sodiumcarbonate pH 9.0 with 2% marvel (0.6+M(9))
2. 3 g/l OMO-MA in 25 mM sodiumcarbonate pH 9.0 with 2% marvel (3+M(9))
3. 6 g/l OMO-MA in 25 mM sodiumcarbonate pH 9.0 with
- 25 2% marvel (6+M(9))
4. 6 g/l OMO-MA in 25 mM sodiumcarbonate pH 9.0 (6 (9))
5. 0.6 g/l LAS in 25 mM sodiumcarbonate pH 9.0 with 2% marvel (0.6L+M(9))
- 30 6. 0.6 g/l LAS in 25 mM sodiumcarbonate pH 10.3 with 2% marvel (0.6L+M(10.3))
7. 3 g/l OMO-MA in water with 2% marvel (3+M(10.3))

The results after three rounds of panning are presented in the following table 8

5

Conditions	round 1	Round 2	round 3
1 0.6+M(9)	1 $10^4$	4 $10^3$	-
2 3+M(9)	1 $10^4$	4 $10^3$	-
3 6+M(9)	5 $10^3$	8 $10^3$	-
4.6 (9)	12	-	-
5, 0.6L+M(9)	1 $10^5$	2 $10^6$	8 $10^4$
6 0.6L+M(10.3)	1 $10^5$	1 $10^6$	8 $10^4$
7 3+M(10.3)	3 $10^5$	7 $10^5$	4 $10^4$
8 PBS+M	1 $10^5$	5 $10^5$	8 $10^4$

As a comparison the results of the panning in PBS (see I.1) and 2% marvel are included.

10 IX.4.2 Screening of free VHH domains produced by selected clones under application conditions.

The fragments indicated in Figure 10, and Table 9 were selected for further investigation

Table 9 Selection conditions

α-RR120 V <sub>H</sub>	Selection conditions
A38, A44	PBS pH 7.4
A306	0.6 g l <sup>-1</sup> LAS pH 9 (25 mM NaCO <sub>3</sub> -buffer)
A307, A308	0.6 g l <sup>-1</sup> LAS pH 10.3 (25 mM NaCO <sub>3</sub> -buffer)
A309	3 g l <sup>-1</sup> OMO pH 10.3 (water)

5 After purification via ion exchange chromatography, the fragments were subjected to an ELISA assay according to the following approach:

An ELISA was performed using serial dilutions (in PBS)  
 10 of the selected α-RR120 antibody fragments resulting in a set of standard dilution curves. The OD 450 nm signal was plotted against the log antibody concentration. The concentration at halve the maximal OD (450) is defined as the midpoint concentration and set as 100 %

15 immunoreactivity for the standard curve.

Subsequently this experiment was repeated using serial dilutions made in product formulations at different concentrations. Again the immunnoreactivity was determined (VHH concentration resulting in half the max OD450 achieved  
 20 in that particular product concentration).

The relative immunoreactivity of the dilution curves in different product concentrations was then expressed as a percentage of the 100 % immunoreactivity as determined from  
 25 the standard curve and represented in figures 8 and 9.

$$\% \text{ immunoreactivity} = \frac{\text{mid-point standard conditions}}{\text{mid-point application conditions}} \times 100$$

5

IX. 4.3 Sequences of VHH domains which bind to RR120  
under application conditions

Figure 10 represents the sequences of VHH domains which  
10 bind to RR120 under the conditions as described above.

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### Claims

1. A method of preparing, storing or using a product comprising active antibodies, wherein said method involves the application of destabilising conditions whereby normally lower eukaryotes or traditional antibodies are killed or inactivated and whereby the active antibodies are VHHs.

2. The use of VHHs in the preparation of products to provide stability of antibody specificity under destabilising conditions whereby normally lower eukaryotes or traditional antibodies are killed or inactivated.

3. Method or use according to claim 1 or 2, wherein the destabilising conditions are such that normally lower eukaryote cells are inactivated with a factor of at least  $10^6$ .

4. Method or use according to claim 1 or 2, wherein the destabilising conditions are such that *Saccharomyces Cerevisae* are inactivated with a factor of at least  $10^6$ .

5. Method or use according to claim 1 or 2, wherein the destabilising conditions are such that normally the immunoreactivity of traditional antibodies is reduced to less than 70%, more preferred less than 10%.

6. Method or use according to claim 1 or 2, wherein the destabilising conditions are such that normally the immunoreactivity of HCGMABs is reduced to less than 70%, more preferred less than 10%.

7. Method or use according to claim 1 or 2 whereby the VHs recognise their binding target e.g. harmful microorganisms or toxins with a binding constant of  $>10^4$ , preferably  $>10^6$ , most preferably  $>10^8$ .
8. Method or use according to claim 1 or 2 whereby the VHs retain their immunoreactivity for at least 70%, upon application of the destabilising conditions.
9. Method or use according to claim 1 or 2, whereby the destabilising conditions involve physical treatments and/or chemical conditions which are applied during preparation, storage or use of the product.
10. Method or use according to claim 1 or 2, whereby the destabilising conditions involve physical treatment selected from one or more of:
- (a) a high temperature treatment, for example at more than 70 °C;
  - (b) ultra high pressure treatment, for example at 250 Mpa or more;
  - (c) irradiation treatment, for example at least 10 kGray gamma radiation;
  - (d) pulse electric field treatment;
- optionally in combination with one or more chemical treatments.
11. Method or use according to claim 1 or 2, whereby the destabilising conditions involve chemical treatment selected from one or more of:

(a) the presence of an effective amount of antimicrobial agent;

(b) the presence of an effective amount of a cell wall degrading enzyme or a redox enzyme,

(c) the presence of solvent materials e.g. alcohol at for example at a level of at least 3 wt%;

(d) the presence of surfactant materials e.g. anionic or zwitterionic detergency materials for example at a level of at least 5wt%

(e) the presence of chaotropic materials

(f) the presence of materials to alter the pH to a value of less than 5 or more than 9; optionally in combination with one or more physical treatments.

12. Method or use according to claim 1 or 2, wherein the selection of suitable VHHs takes place under destabilisation conditions.

13. Method or use according to claim 1 or 2, wherein the selection of suitable VHHs and at least one of the group of manufacture, storage or use takes place under destabilising conditions.

14. Method or use according to claim 1 or 2, wherein the manufacture of the product involves the application of destabilising conditions.

15. Method or use according to claim 1 or 2 wherein the storage of the product involves the application of destabilising conditions.

16. Method or use according to claim 1 or 2, wherein the use of the product involves the application of destabilising conditions.

17. Method or use according to claim 1 or 2, wherein the product is a food product selected from the group ice-cream, oil based products such as margarines, oils, mayonnaise, dressings, soups, sauces, soft drinks, tea based drinks and meals.

18. Method according to claim 1 or 2, wherein the product is a personal care product selected from the group of cleaning products like soap bars, washing liquids, gels, laundry detergents, deodorants, creams, lotions and shampoo.

19. Method according to claim 1 or 2, wherein the product is an animal feed product.

20. Method to prepare a product comprising VHHs, comprising the steps of:

- a. selection of suitable VHHs by screening under conditions which are equal or similar to the conditions of the product;
- b. incorporating the selected VHHs in the product.

21. Product obtainable by a method of claim 20.

22. Product according to claim 21 being a food product or a personal care product.

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Fig.1.

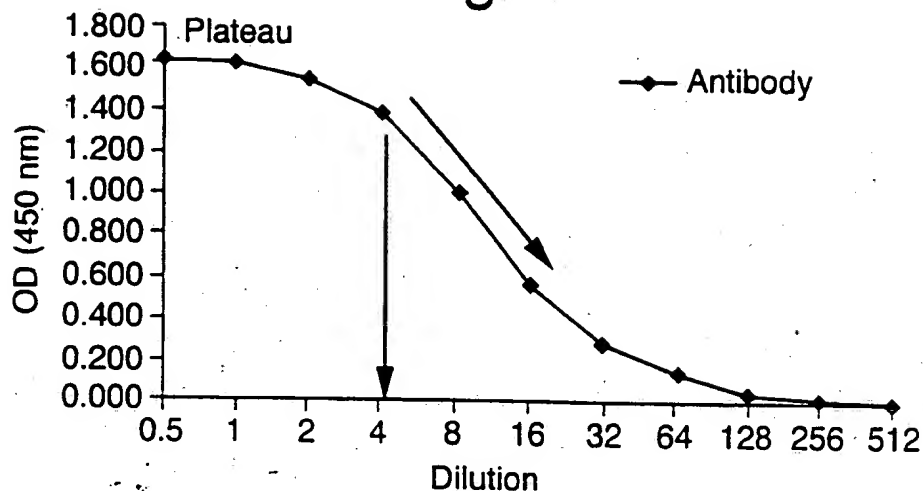


Fig.5.

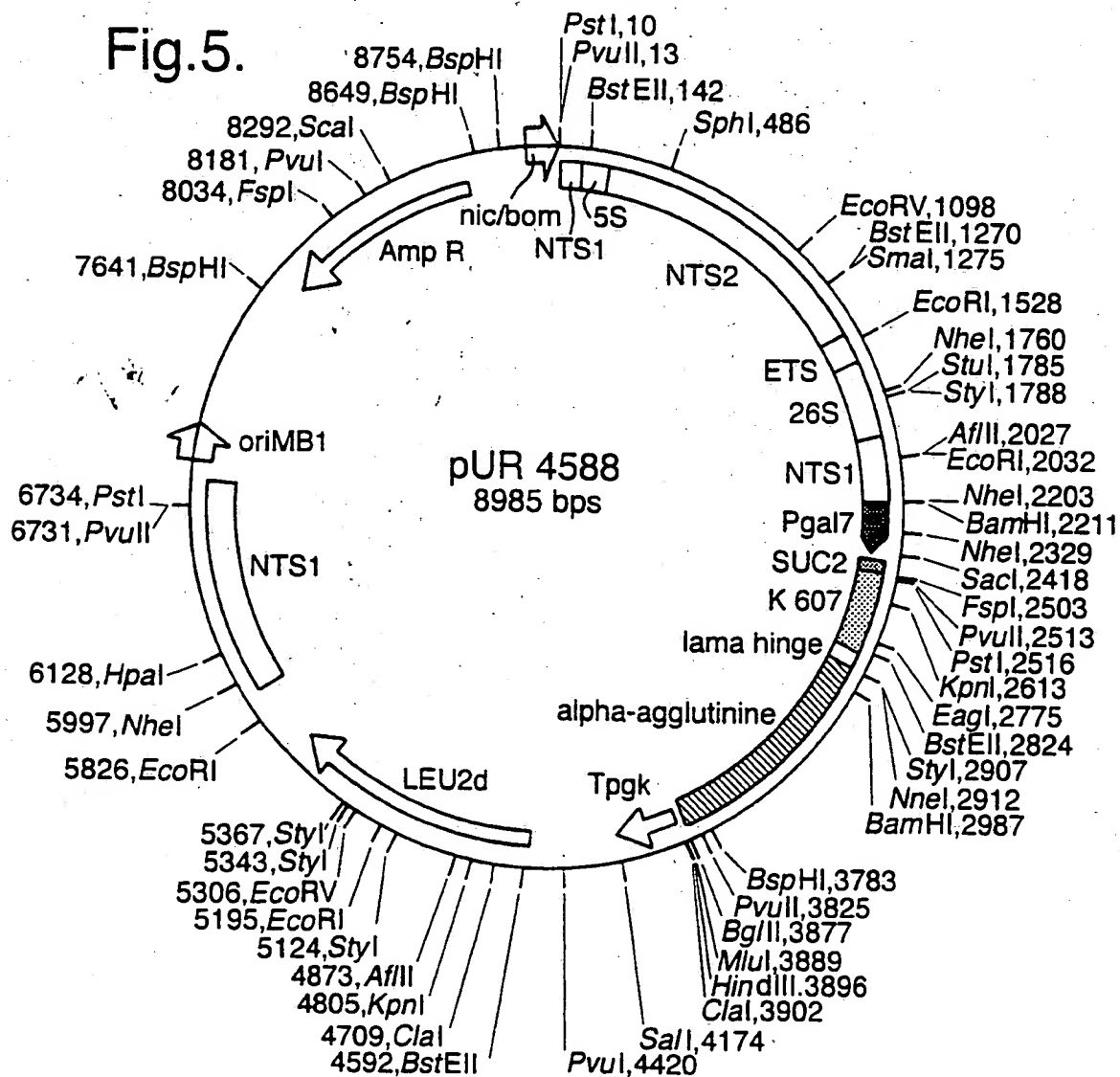


Fig.2A.

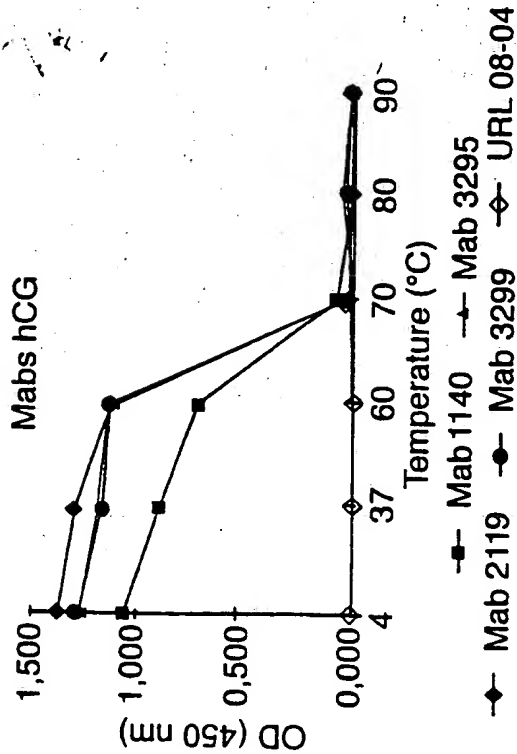


Fig.2B.

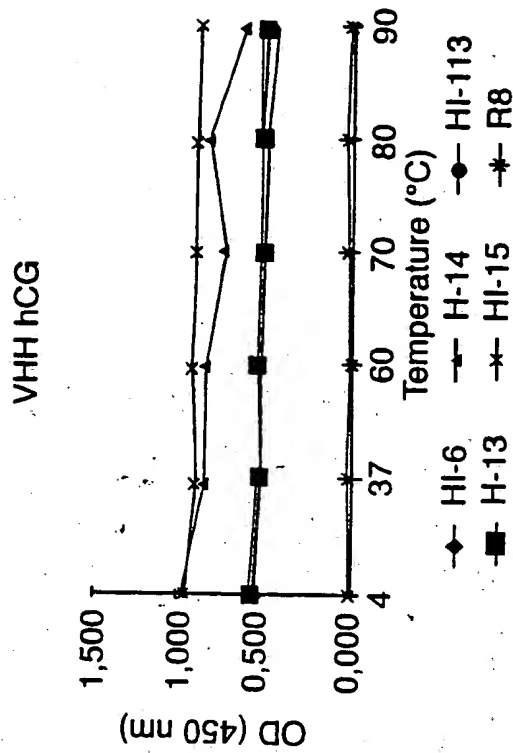


Fig.2C.

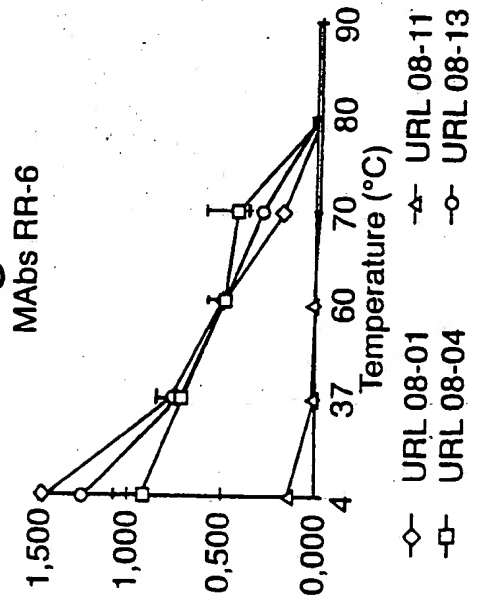


Fig.2D.

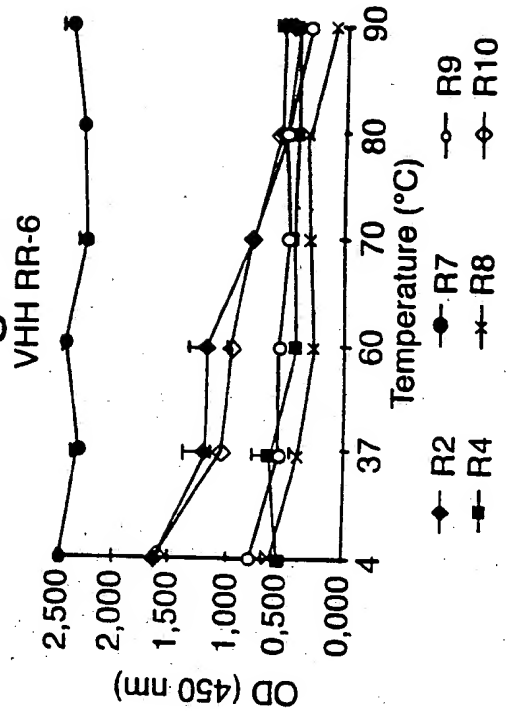




Fig.3A.

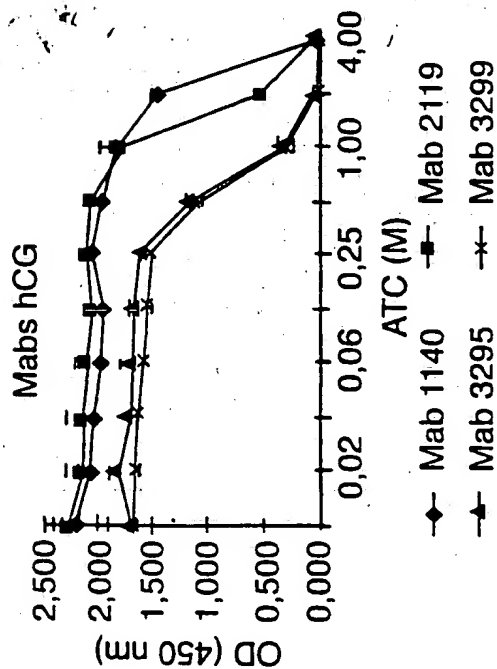


Fig.3B.

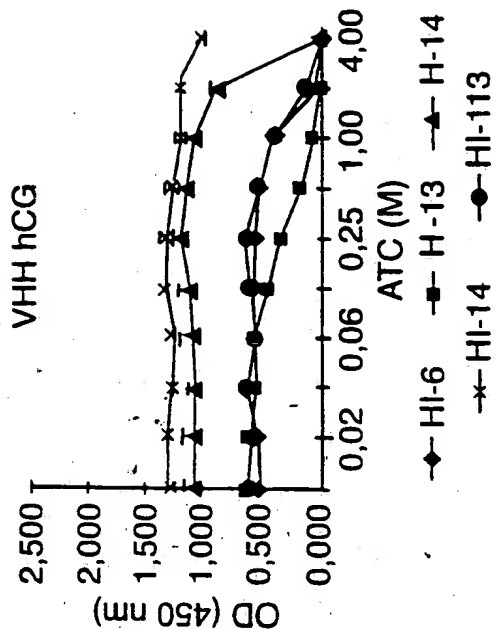


Fig.3C.

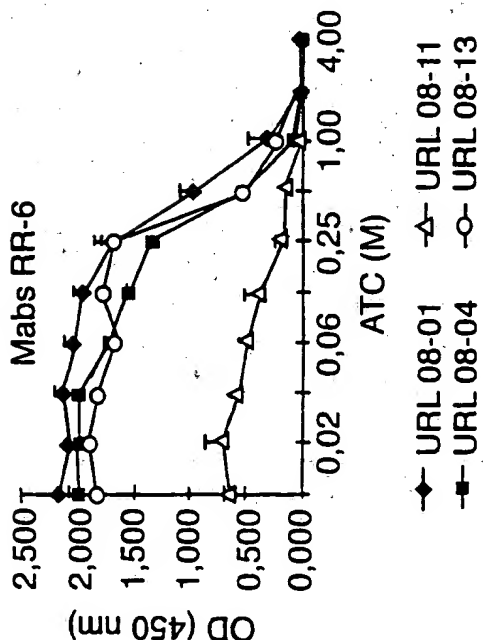


Fig.3D.

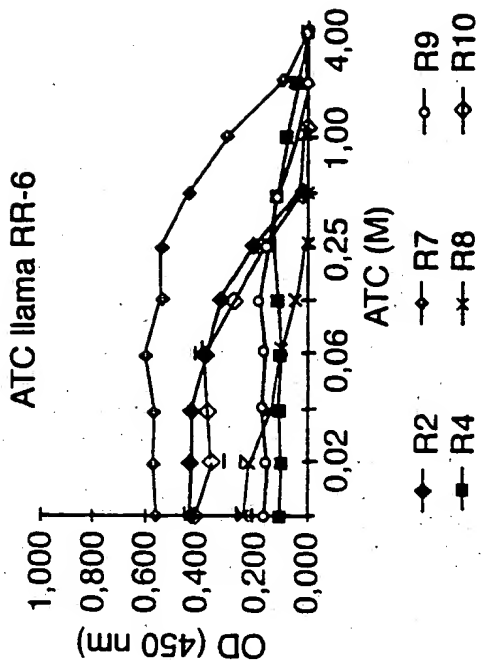


Fig.4.

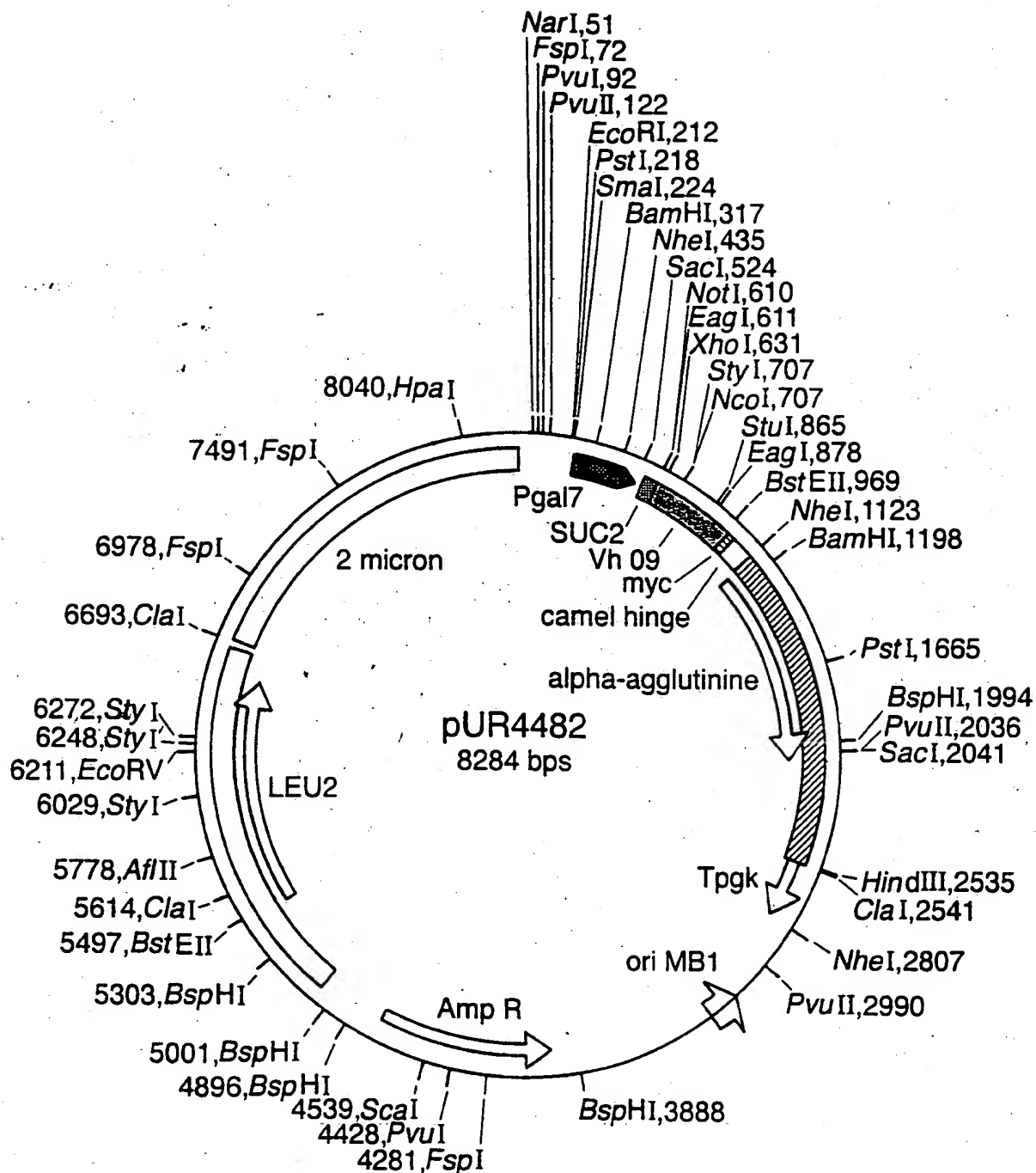


Fig.6.

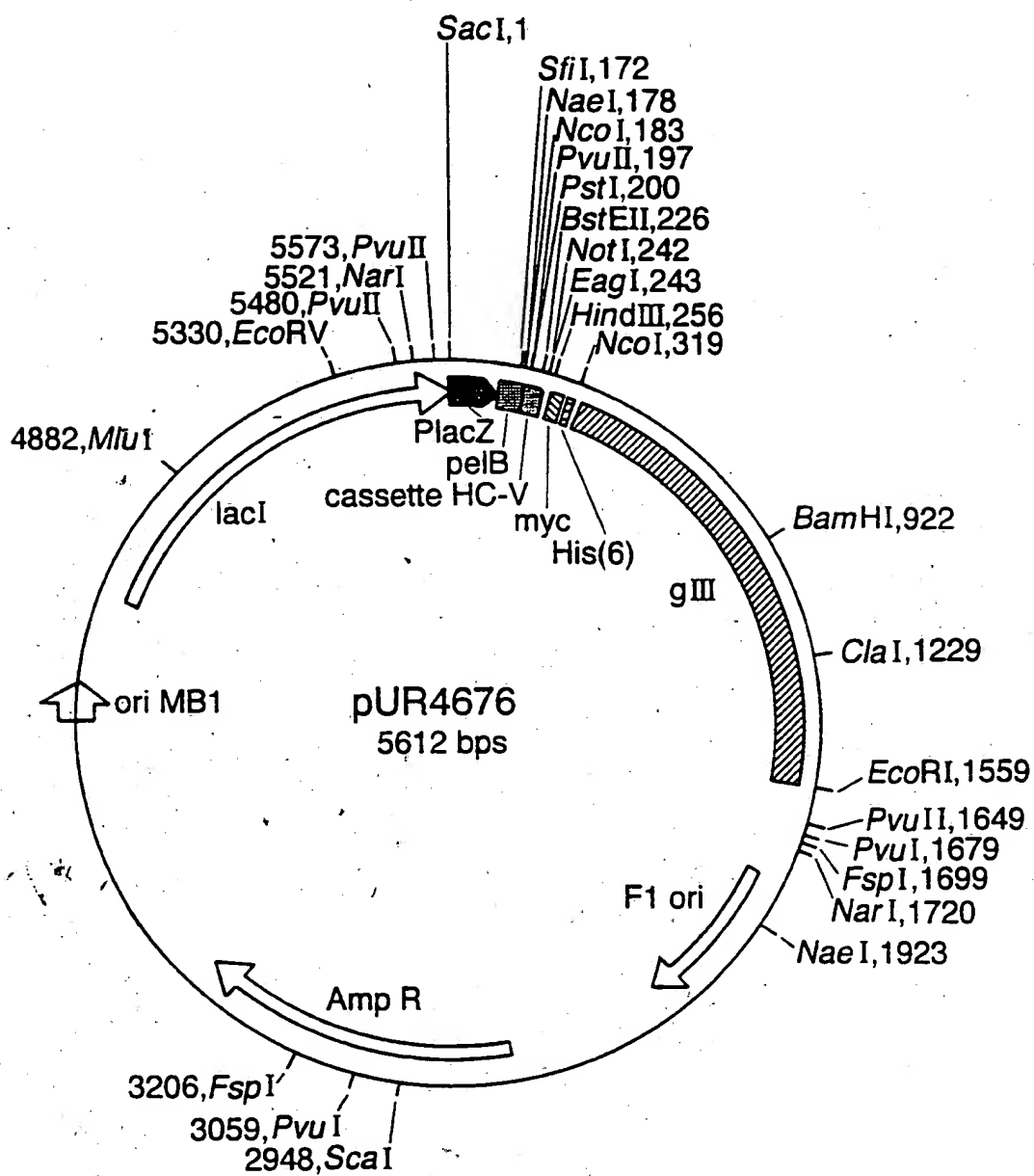
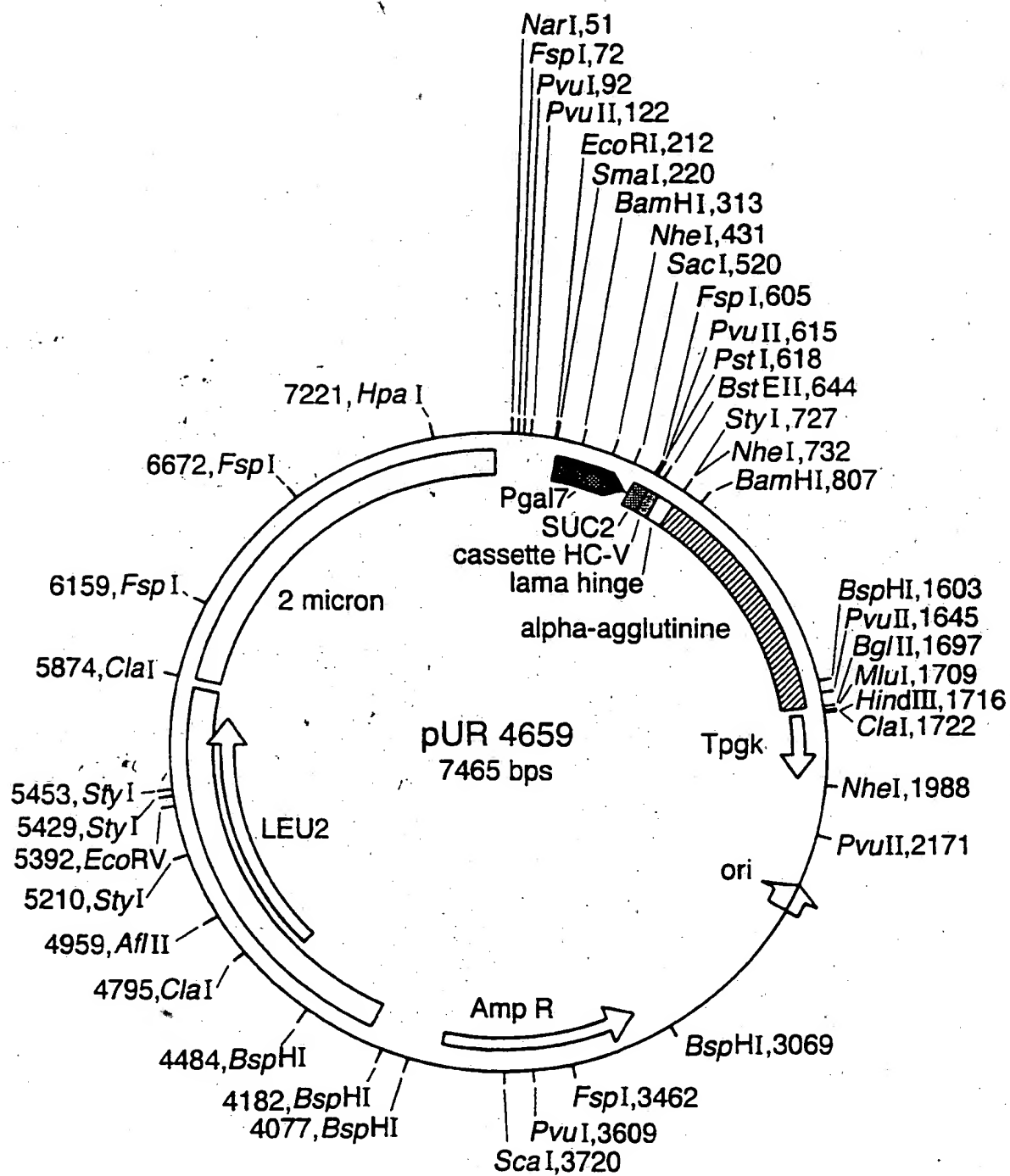


Fig.7.



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Fig.8.

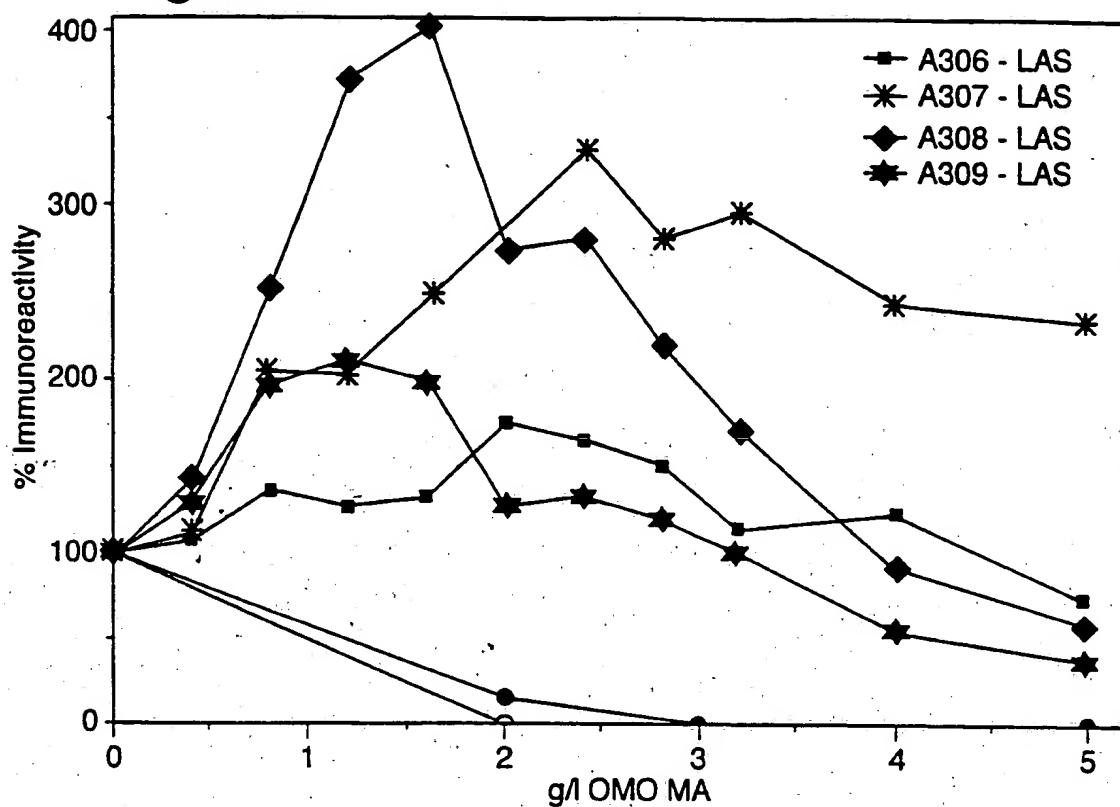


Fig.9.

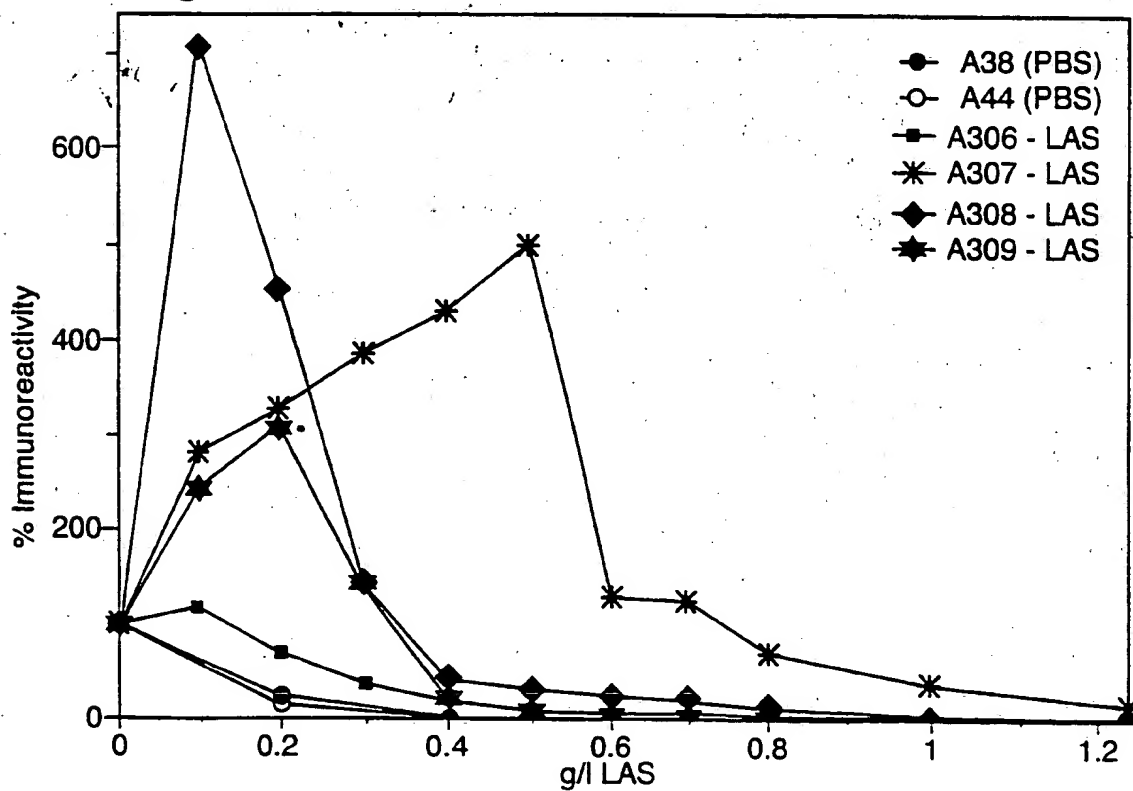


Fig.10.

	frame I		cdr I	frame II
	10 20 30			40
RR120_38	QVQLQESGGGLVQAGDSLRLSCEASGPTFS		RYAFG	WFRQTPGQEREFVG
RR120_44	QVQLQESGGGLVHAGGSLRLSCAASGRIFR		IDSMA	WYRQTPGKQRELVA
RR120_306	QVQLQESGGGLVQAGGSLRLSCVASGNDFS		IYDIG	WYRQAPGKPREFVA
RR120_307	QVQLQESGGGLVQAGDSLRLSCVASGNDFS		IYDIG	WYRQAPGNPREFVA
RR120_308	QVQLQESGGGLVQAGGSLRLSCVASGNDFS		IYDIG	WYRQAPGKPREYVA
RR120_309	QVQLQQSGGGVHAGGSLRLSCVASGRIFR		IDEMS	WHRQTPGKQRELVA
	cdr II	frame III		
	50 60	70 80 90		
RR120_38	AMTWRGGLTSVVADSVKG	RFTIFRDMTRNMMWLQMNDLKAGDSAVYYCAA		
RR120_44	TIT--DGMTNYADSVRG	RFTISRDKAKNTVYLOMNILKPEDTAVYYCNA		
RR120_306	AIG--RGGYTNIDASVKG	RFTISRDNKNTVYLOMDTLKPEDTAVYSCAA		
RR120_307	AIG--RGGYTNIDASVKG	RFTISRDNKNTVYLOMNTLKPEDTAVYSCAA		
RR120_308	AVG--KGGYTNIAASVKG	RFTISRDNKNTVYLOMNTLKPEDTAVYSCAA		
RR120_309	SMS--IDGVAKYADSVKG	RFTISRDNKNTVYLOMNFLKHEDTAVYYCNA		
	cdr III	frame IV		
	100	110		
RR120_38	RPRGSLYSEDSY--DY	WGQGTQVTVSS		
RR120_44	RGPYSRGS-----GP	WGQGTQVTVSS		
RR120_306	AKRYGSGRLDDITRYNY	WGQGTQVTVSS		
RR120_307	AQRYGPGRLNDISRYNY	WGQGTQVTVSS		
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RR120_309	RGPYSRGS-----GP	WGQGTQVTVSS		

## SEQUENCE LISTING

<110> Unilever N.V. / Unilever PLC

5 <120> Use of antibodies

<130> T.7062 (V)

<140>

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 Ala Met Thr Trp Tyr Arg Gln Ala Pro Gly Lys Ser Arg Glu Tyr Val  
 35 40 45  
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 Ala Arg Ile Phe Phe Ser Gly Gly Thr Asn Tyr Ala Asp Ser Val Lys  
 50 55 60  
 Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Met Tyr Leu  
 40 65 70 75 80  
 Gln Met Asn Asp Leu Lys Arg Glu Asp Thr Ala Val Tyr Tyr Cys Asn  
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 10 Ala Met Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Phe Val  
 35 40 45  
 Ala Thr Val Ser Arg Gly Gly Gly Ser Thr Tyr Tyr Ala Asp Ser Val  
 50 55 60  
 15 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr  
 65 70 75 80  
 Leu Gln Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Ala Tyr Tyr Cys  
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 40 65 70 75 80  
 Trp Leu Gln Met Asn Asp Leu Lys Ala Gly Asp Ser Ala Val Tyr Tyr  
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                     35                    40                    45  
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                     20                    25                    30  
 50 Asp Ile Gly Trp Tyr Arg Gln Ala Pro Gly Lys Pro Arg Glu Phe Val  
                     35                    40                    45

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50 55 60

5 Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr Leu  
65 70 75 80

Gln Met Asp Thr Leu Lys Pro Glu Asp Thr Ala Val Tyr Ser Cys Ala  
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Asp Ile Gly Trp Tyr Arg Gln Ala Pro Gly Asn Pro Arg Glu Phe Val  
35 40 45

35 Ala Ala Ile Gly Arg Gly Gly Tyr Thr Asn Ile Asp Ala Ser Val Lys  
50 55 60

Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr Leu  
40 65 70 75 80

Gln Met Asn Thr Leu Lys Pro Glu Asp Thr Ala Val Tyr Ser Cys Ala  
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Asp Ile Gly Trp Tyr Arg Gln Ala Pro Gly Lys Pro Arg Glu Tyr Val  
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Ala Ala Val Gly Lys Gly Gly Tyr Thr Asn Ile Ala Ala Ser Val Lys  
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Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr Leu  
65 70 75 80

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Gln Met Asn Thr Leu Lys Pro Glu Asp Thr Ala Val Tyr Ser Cys Ala  
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Ser Tyr Trp Gly His Gly Thr Gln Val Thr Val Ser Ser  
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Glu Met Ser Trp His Arg Gln Thr Pro Gly Lys Gln Arg Glu Leu Val  
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Ala Ser Met Ser Ile Asp Gly Val Ala Lys Tyr Ala Asp Ser Val Lys  
50 55 60

5 Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr Leu  
65 70 75 80

Gln Met Asn Phe Leu Lys His Glu Asp Thr Ala Val Tyr Tyr Cys Asn  
85 90 95

10 Ala Arg Gly Pro Tyr Ser Arg Gly Ser Gly Pro Trp Gly Gln Gly Thr  
100 105 110

Gln Val Thr Val Ser Ser  
15 115

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 99/01678

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 6 C07K16/00 A61K39/395 C07K1/14 A23L1/30 A23L1/305 C11D7/00 A61K7/06 A61K7/32 A61K7/48		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 6 A23K C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 25591 A (UNILEVER) 10 November 1994 (1994-11-10) cited in the application examples claims	1-16
A	WO 94 18330 A (UNILEVER) 18 August 1994 (1994-08-18) cited in the application examples claims	1-16
A	EP 0 739 981 A (VRIJE UNIVERSITEIT BRUSSEL) 30 October 1996 (1996-10-30) claims	1-16
-/-		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "A" document member of the same patent family		
Date of the actual completion of the international search  23 August 1999		Date of mailing of the international search report  30/08/1999
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer  Nooij, F

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 99/01678

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 29457 A (UNILEVER) 22 December 1994 (1994-12-22) claims	1-16
A	J. DAVIES ET AL.: "Single antibody domains as small recognition units: design and in vitro antigen selection of camelized, human Vh domains with improved protein stability." PROTEIN ENGINEERING, vol. 9, no. 6, June 1996 (1996-06), pages 531-537, XP002080377 Oxford, GB the whole document	1-16

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 99/01678

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1, 3-19  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 1, 3-19 (all partially, as far as an in vivo method is concerned) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## B x II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 99/01678

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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